The evaluation of lysozyme under winemaking conditions

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

The use of lysozyme in oenology is a relative new concept. It forms with bacteriocins and glucose oxidase part of biopreservation of wine. Biopreservation refers to natural substances contributing to the stability of food and beverages. Lysozyme was approved in 2000 by the *Office International de la Vigne et du Vin* (OIV) for usage in wine. Lysozyme is commercially extracted from hen egg white and inhibits Gram-positive microorganisms such as lactic acid bacteria (LAB). Lysozyme's mode of action is through degradation of peptidoglycan and cleaves the β-(1-4)-glycosidic bond between *N*-acetylmuramic acid (MurNAc) and *N*-acetyl-D-glucosamine (GlcNAc) in the polysaccharide, which form peptidoglycan, the essential constituent of bacterial cell walls. The control of LAB during winemaking is especially important due to spoilage LAB that can produce biogenic amines, off-flavours, volatile acidity, ropiness, ethyl carbamate, bitterness, mannitol, geranium tone and they can also play a role in stuck alcoholic fermentation. One of the primary agents used for the control of microorganisms is sulphur dioxide (SO_2) due to its anti-oxidative and antimicrobial functions. However, the action of SO_2 is pH dependant and consumer demand has increased for lowered SO_2 levels in wine.

This study therefore focused on the evaluation of lysozyme under South African winemaking conditions by investigating the influence of lysozyme on different strains of LAB and the effect on the alcoholic fermentation tempo (AFT) during small-scale fermentations. Secondly the effect of lysozyme on acetic acid bacteria (AAB) and LAB numbers during Pinotage, Cabernet Sauvignon and Shiraz red wine vinifications were evaluated. Other wine parameters monitored in the red wine vinifications included volatile components, biogenic amine levels, colour and total phenol content.

It was shown that lysozyme was effective in lowering, or completely inhibiting, LAB growth with the exception of a few strains, thus indicating differences in sensitivity towards lysozyme between species and strains. Sensitivity to lysozyme treatments was seen for strains of *L. nagelii*, *L. pentosus*, *L. vermiforme*, *L. paraplantaum*, *L. hilgradii*, *L. plantarum*, *L. paracasei* and *L. buchneri*. Strains that did not show positive growth during the alcoholic fermentation (AF) included those of *L. plantarum*, *L. fermentum*, *Pediococcus acidilactici*, *L. pentosus* and *Leuconostoc mesenteroides*. This study showed that wine isolated *Lactobacillus* strains of *L. brevis*, *L. buchneri* and *L. paracasei* are more resistant to lysozyme concentrations during a controlled small-scale AF. No effect was observed for AFT. Furthermore, lysozyme treatment did not result in an increase in AAB growth during AF, however, in some cases resulted in lower AAB numbers for lysozyme treated wines. Differences in LAB and AAB numbers could be seen between the tested cultivars. No conclusion could be drawn from this study on the effect on the volatile compounds and biogenic amine and needs further investigation. Lysozyme treatment did not have any effect on colour or total phenol content of red wine.

OPSOMMING

Die gebruik van lisosiem in wynkunde is 'n redelike nuwe konsep. Lisosiem vorm saam met bakteriosiene en glukose oksidase deel van biopreservering strategieë vir wyn. Biopreservering verwys na natuurlike middels wat bydra tot die stabiliteit van voedsel- en drankprodukte. Lisosiem is in 2000 goedgekeur deur die *Office International de la Vigne et du Vin* (OIV) vir die gebruik in wyn. Kommersieël word lisosiem uit eierwit geëkstraheer en gebruik om Gram-positiewe organismis soos melksuurbakterieë (MSB) te inhibeer. Lisosiem se werking is deur die afbraak van peptidoglukaan en breek die β-(1-4)-glikosidiese verbinding tussen *N*-asetiel muramiensuur (MurNAc) en *N*-asetiel-D-glukosamien (GlcNAc) in die polisakkaried wat peptidoglukaan vorm, die noodsaaklike bousteen waaruit bakteriële selwande bestaan. Die beheer van MSB in wyn is veral belangrik vanweë die vermoë van bederf MSB om biogene amiene, afgeure, vlugtige suur, draadagtigheid, etielkarbamaat, bitterheid, mannitol en malva-agtigheid te vorm en MSB kan ook 'n rol speel by slepende/gestaakte alkoholiese gistings. Een van die belangrikste middels om mikroörganismes te beheer is swaweldioksied (SO_2) vanweë die anti-oksiderende en antimikrobiese werking van SO_2. Die werking van SO_2 is pH afhanklik en hedendaagse verbruikerstendense neig na verlaagde SO_2 vlakke in wyn.

Hierdie studie het dus gefokus op die evaluasie van lisosiem onder Suid-Afrikaanse wynmaak toestande deur die invloed van lisosiem op verskillende rasse van MSB en gevolglik op die alkoholiese fermentasie tempo (AFT) te evalueer tydens kleinskaalse fermentasies. Tweedens is die effek van lisosiem op asynsuurbakterieë (ASB) en MSB getalle tydens Pinotage, Cabernet Sauvignon en Shiraz rooiwynmakery getoets. Ander parameters wat ook gemonitor is sluit vlugtige komponente, biogeniese amien vlakke, kleur en totale fenol vlakke in.

Die studie het getoon dat lisosiem effektief was in die verlaging of algehele inhibisie van MSB groei met die uitsluiting van 'n paar rasse wat dus wys op sensitiwiteits verskille tussen rasse en spesies. Sensitiwiteit vir lisosiem behandeling is gesien vir rasse van *L. nagelii*, *L. pentosus*, *L. vermiforme*, *L. paraplantarum*, *L. hilgardii*, *L. plantarum*, *L. paracasei* en *L. buchneri*. Rasse wat nie positiewe groei getoon het tydens die alkoholiese fermentasie nie sluit die van *L. plantarum*, *L. fermentum*, *Pediococcus acidilactici*, *L. pentosus* en *Leuconostoc mesenteroides*. Hierdie studie het getoon dat meestal wyngeïsoleerde rasse van *Lactobacillus* in *L. brevis*, *L. buchneri* en *L. paracasei* meer weerstandbiedend is teen lisosiem toevoegings gedurende 'n beheerde kleinskaalse fermentasie. Geen effek op die AFT is waargeneem nie. Verder het lisosiem toediening nie gelei tot 'n verhoging in ASB getalle tydens alkoholiese fermentasie nie en wel in sekere gevalle laer ASB getalle vir lisosiem behandelde wyne getoon. Verskille in terme van MSB en ASB getalle kon gesien word tussen kultivars. Geen gevolgtrekking kon gemaak word in terme van die invloed van lisosiem op die vlugtige komponente en biogeniese amiene en benodig verdere navorsing. Lisosiem toediening het geen effek gehad op kleur en totale fenol inhoud van rooiwyn nie.

This thesis is dedicated to parents, Owen and Johanna.
Hierdie tesis is my aan ouers, Owen en Johanna, opgedra.

BIOGRAPHICAL SKETCH

Riaan Frederick Wassüng was born in Parow, South Africa on 21 February 1978. He attended Horison Primary School (Gauteng), Hangklip Primary School and matriculated at Hangklip High School, Queenstown in 1996.

Riaan entered Stellenbosch University (Stellenbosch) and obtained a BScAgric degree in Viticulture and Oenology in 2001. In 2002, he enrolled for an MScAgric degree in Oenolgy at the same University.

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PREFACE

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the *South African Journal of Enology and Viticulture* to which Chapter 3 will be submitted for publication.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**
The microbial stabilisation and preservation of wine

Chapter 3 **RESEARCH RESULTS**
The evaluation of lysozyme under winemaking conditions

Chapter 4 **GENERAL DISCUSSION AND CONCLUSIONS**

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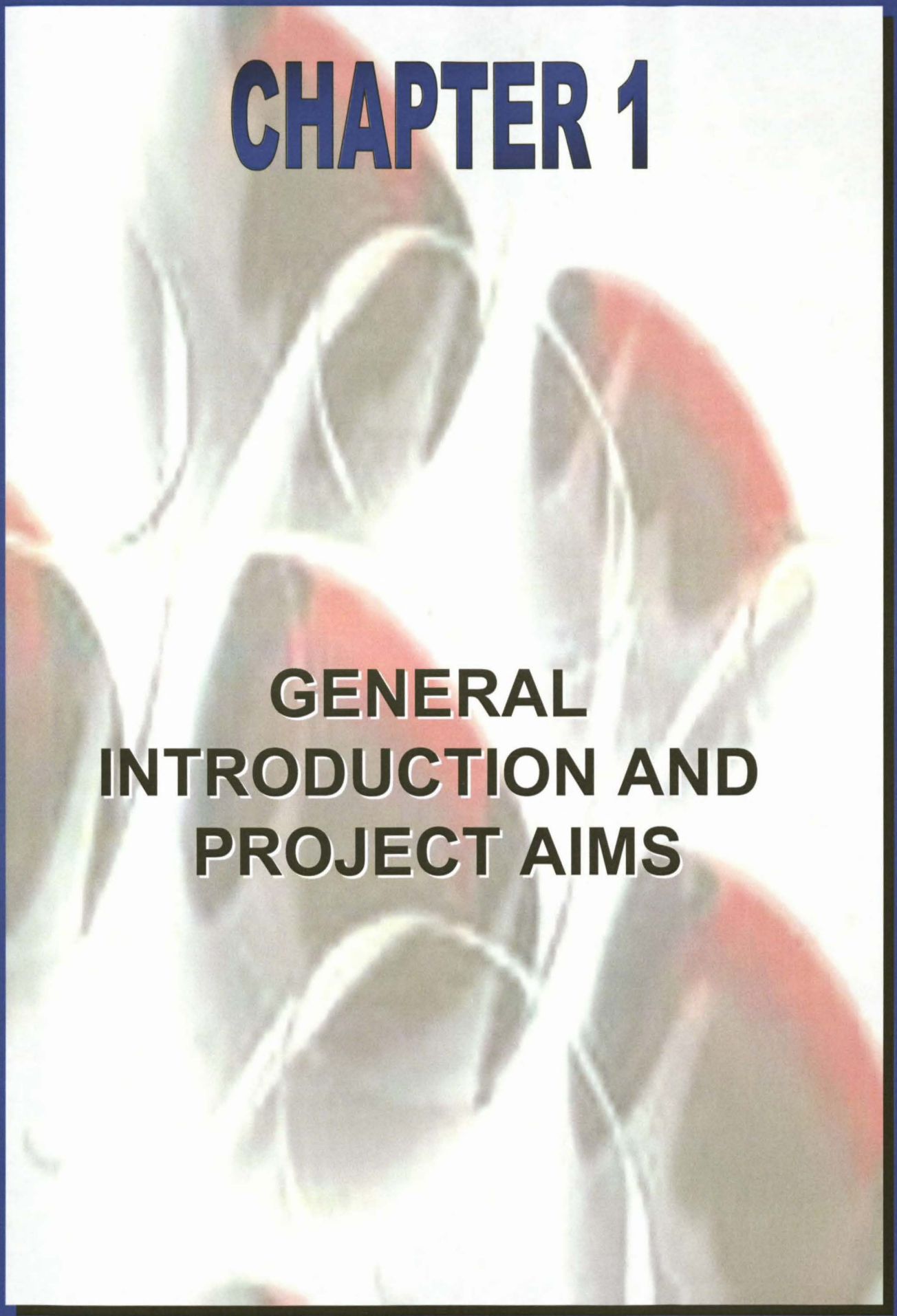
CHAPTER 1



GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 PRESERVATION OF WINE

The South African wine industry's history dates back to 2 February 1659 when Jan van Riebeeck made the first wine from locally harvested *Vitis vinifera* grapes (Thom, 1958). Since then, the wine industry underwent major changes. Wine styles differ and wine prices are now more competitive on the international market and millions of Rands worth of wine are exported each year to foreign markets.

Globally, consumer awareness of preservatives in foods and beverages has increased with preference for more 'green' products that are healthier. In this regard it has been shown that quality wine, and especially red wine, have several health benefits if consumed in moderate quantities. This is due to the polyphenolic compounds in red wine, which originates from the skins, seeds, stems and oak- derived products (Rice-Evans *et al.*, 1995, 1997; German & Walzem, 2000).

The winemaking process consists of several phases, which begins with grapes in the vineyard and finishes with the bottling of the final product. The three groups of microorganisms involved in winemaking include yeasts which can be divided into wild yeasts (Non-*Saccharomyces* species) (Ribéreau-Gayon, 1985; Boulton *et al.*, 1996; Fleet, 1998; Pretorius *et al.*, 1999; Zoecklein *et al.*, 1995) and *Saccharomyces* yeasts (Du Toit & Pretorius, 2000), lactic acid bacteria (LAB) (species of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*) (Lonvaud-Funel, 1999; Ribéreau-Gayon *et al.*, 2000a) and acetic acid bacteria (species of *Gluconobacter*, *Acetobacter* and *Gluconoacetobacter*) (Joyeux *et al.*, 1984a, b; Drysdale & Fleet, 1988; Yamada *et al.*, 1997; Ruiz *et al.*, 2000). Effective control of these organisms during alcoholic fermentation and maturation is essential for the desired quality level of wine.

In this regard, sulphur dioxide (SO_2) has long been used due to its dual antimicrobial and anti-oxidative functions in wine (Ribéreau-Gayon *et al.*, 2000a). In wine and juices, SO_2 is very effective in inhibiting all wine-associated microorganisms. However, different microorganisms differ in their sensitivity to SO_2. At pH levels lower than 3.5, SO_2 is very effective due to the presence of the free molecular fraction, which can exert antimicrobial activity. However, at higher pH values (> 3.5) the antimicrobial ability of SO_2 diminishes, due to the lesser amount of molecular SO_2, which can result in spoilage or oxidation (Usseglio-Tomasset, 1992; Zoecklein *et al.*, 1995; Ribéreau-Gayon *et al.*, 2000a, b). Higher pH wines are usually found in warmer wine-producing countries such as South Africa, or it can be as a result of high temperatures during the different phenological stages of the vine and/or grapes. Potassium plays a vital role in high pH wines (Winkler *et al.*, 1974; Boulton, 1980; Iland, 1988). Therefore, other preservatives have been tried and tested under oenological conditions as an alternative, but only a few are acceptable for the use in wine (Ough, 1975; Stead, 1993; 1994; Zoecklein *et al.* 1995; Brul & Coote, 1999).

In South Africa, by law, producers may only use the following antimicrobials: SO_2 (ammonium bisulphite, potassium- and sodium metabisulphite), dimethyl dicarbonate (DMDC, Legal limit (LL.) < 100 mg/L), natamycin (LL. < 30 mg/L), sodium benzoate (analysed as benzoic acid, LL. < 200 mg/L), sorbic acid (LL. < 200 mg/L), potassium benzoate (analysed as sorbic acid, LL. < 200 mg/L) and citric acid (http://www.sawis.co.za). In this regard, SO_2 is the only preservative with an anti-oxidative function as well. All other preservatives degrade more rapidly over time, for instance LAB converting fumaric acid into malic acid (Zoecklein *et al.*, 1995), DMDC reacting with alcohols to form breakdown products of methanol, carbon dioxide and methyl alkyl carbonates (Peterson & Ough, 1979) to name a few examples. For this reason, certain preservatives such as benzoic acid and sorbic acid are mainly used in fruit juices and non-alcoholic beverages. Countries differ in their legislation for certain antimicrobials used in wine, for instance the use of natamycin in wine destined for export to the European Union (EU) countries is prohibited.

In wine, LAB are responsible for the conversion of L- malic acid (malate) into L- lactic acid (lactate) and carbon dioxide (CO_2), known as malolactic fermentation (MLF) (Lonvaud-Funel, 1999; Bartowsky & Henschke, 2004). This process stabilises the wine against any further reduction of malate. Other positive contributions include changes in aroma profiles (mainly diacetyl), increased complexity and increased mouthfeel of wine (Edwards *et al.*, 1991; Nielsen *et al.*, 1996; Maicas *et al.*, 1999; Bartowsky & Henschke, 2004). *Oenococcus oeni* [formerly known as *Leuconostoc oenos* (Dicks *et al.*, 1995)] is the preferred species to conduct MLF.

On the other hand, species of *Lactobacillus*, *Pediococcus* and *Leuconostoc* have been implicated in spoilage of wine and are thus mostly unwanted at any stage of winemaking. The ability of these spoilage LAB species to produce compounds that can negatively affect the sensory and physical attributes of wine is the main reasons why *O. oeni* is preferred. These effects can be in the form of acetic acid production (increased volatile acidity levels) (Maicas *et al.*, 1999; Du Toit & Pretorius, 2000), ropiness (Lonvaud-Funel, 1999; Du Toit & Pretorius, 2000), biogenic amine production (Guerrine *et al.*, 2001), ethyl carbamate (Lonvaud-Funel, 1999; Du Toit & Pretorius, 2000; Uthurry *et al.*, 2006), potential sluggish and/or stuck alcoholic fermentations (Edwards *et al.*, 1998, 1999), bitterness, mannitol production and geranium tone (Du Toit & Pretorius, 2000).

Lysozyme, an enzyme commercially extracted from hen egg white, have been used with great success in the cheese industry to combat a problem known as 'butyric late blowing', caused by *Clostridium tyrobutyricum* (Proctor & Cunningham, 1988; Brul & Coote, 1999; http://www.lysozyme.com). Lysozyme only affects Gram-positive organisms, due to the composition of their cell membrane (Ohno & Morrison, 1989). Thus, lysozyme exerts bacteriolytic activity towards LAB (Board, 1995). Due to consumer demands for lowered sulphite levels in wines, the use of lysozyme in oenology has been approved by the *Office International de la Vigne et du Vin* (OIV) in 2000 and is now legal in almost all

wine producing countries (Bartowsky, 2003). Therefore, lysozyme can be used in wine for the control of LAB.

1.2 SPECIFIC PROJECT AIMS

This study forms part of a crucial research project on the biopreservation of wine in the Institute for Wine Biotechnology. Biopreservation refers to the use of natural substances to promote the preservation of wine. As mentioned before, the control of spoilage microorganisms for the desired quality level of wine is crucial. In the fight against these spoilage microorganisms the focus is placed, in this case, on the use of lysozyme as biopreservative. In the early days, fresh egg white was used for the fining of red wines, thus unsuspectingly adding a small part of active lysozyme. Lysozyme is intensively used in the cheese industry to control spoilage by LAB. However, the use of pure lysozyme in oenology is a relatively new concept, by means of physical or direct addition. The specific aims of this study were therefore:

(i) to examine the influence of three different lysozyme concentrations on the growth of wine-associated lactic acid bacteria during a controlled alcoholic fermentation (AF);
(ii) to investigate if lysozyme had any effect on the alcoholic fermentation tempo;
(iii) to determine the effect of lysozyme on acetic acid bacteria and lactic acid bacteria numbers during red wine vinifications;
(iv) to evaluate the influence of lysozyme on the colour and phenolic content of red wine if it is added at the start of AF;
(v) the effect of lysozyme on the volatile components produced during the AF and MLF.

1.3 LITERATURE CITED

Bartowsky, E., 2003. Lysozyme and winemaking. Aust. & NZ Grapegrower & Winemaker: 473a, 101-104.

Bartowsky, E.J. & Henschke, P.A., 2004. The 'buttery' attribute of wine-diacetyl-desirability, spoilage and beyond. Int. J. Food Microbiol. 96, 235-252.

Brul, S. & Coote, P., 1999. Preservative agents in foods: mode of action and microbial resistance mechanisms. Int. J. Food Microbiol. 50, 1-17.

Board, R.G., 1995. Natural antimicrobials from animals. In: Gould, G.W. (ed.). New Methods of Food Preservation. Blackie Academic & Professional, UK. pp. 40-57.

Boulton, R., 1980. The relationship between total acidity, titratable acidity and pH in grape tissue. Vitis 19, 113-120.

Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E., 1996. Principles and Practices of Winemaking. Chapman & Hall, New York, NY. pp. 244-273.

Dicks, L.M.T., Dellaglio, F. & Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig] gen. nov., comb. nov. Int. J. Syst. Bacteriol. 45, 395-397.

Drysdale, G.S. & Fleet, G.H., 1988. Acetic acid bacteria in winemaking: A Review. Am. J. Enol. Vitic. 39, 143-154.

Du Toit, M. & Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - A Review. S. Afr. J. Enol. Vitic. 21, Special Issue, 74-96.

Edwards, C.G., Jensen, K.A., Spayd, S.E. & Seymor, B.J., 1991. Isolation and characterization of native strains of *Leuconostoc oenos* from Washington state wines. Am. J. Enol. Vitic. 42, 219-225.

Edwards, C.G., Haag, K.M. & Collins, M.D., 1998. Identification of some lactic acid bacteria associated with sluggish/stuck fermentations. Am. J. Enol. Vitic. 49, 445-448.

Edwards, C.G., Haag, K.M., Semon, M.J., Rodrigues, A.V. & Mills, J.M., 1999. Evaluation of processing methods to control the growth of *Lactobacillus kunkeei*, a micro-organism implicated in sluggish alcoholic fermentations of grape musts. S. Afr. J. Enol. Vitic. 20, 11-18.

Fleet, G.H., 1998. The Microbiology of Alcoholic Beverages. In: Wood, B.J.B. (ed). Microbiology of Fermented Foods, vol. 1. Blackie Academic & Professional, London. pp. 217-262.

German, J.B. & Walzem, R.L., 2000. The health benefits of wine. Annu. Rev. Nutr. 20, 561-593.

Guerrini, S., Mangani, S., Granchi, L. & Vincenzini, M., 2001. Biogenic amine production by *Oenococcus oeni*. Curr. Microbiol. 44, 374-378.

Iland, P.G., 1988. Grape berry ripening: the potassium story. Aust. Grapegrower Winemaker Jan. 22-24.

Joyeux, A., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984a. Evolution of acetic acid bacteria during fermentation and storage of wine. Appl. Environ. Microbiol. 48, 153-156.

Joyeux, A., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984b. Metabolism of acetic acid bacteria in grape must: consequences on alcoholic and malolactic fermentation. Sci. Aliment. 4, 247-255.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek 76, 317-331.

Nielsen, J.C., Prahl, C. & Lonvaud-Funel, A., 1996. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. Am. J. Enol. Vitic. 47, 42-48.

Maicas, S., Gil, J., Pardo, I. & Ferrer, S., 1999. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food Res. Int. 32, 491-496.

Ohno, N. & Morrison, D.C., 1989. Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme activity. J. Biol. Chem. 264, 4434-4441.

Ough, C.S., 1975. Dimethyldicarbonate as a wine sterilant. Am. J. Enol. Vitic. 26, 130-133.

Peterson, T.W. & Ough, C.S., 1979. Dimethyldicarbonate reaction with higher alcohols. Am. J. Enol. Vitic. 30, 119-123.

Pretorius, I.S., Van der Westhuizen, T.J. & Augustyn, O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. S. Afr. J. Enol. Vitic. 20, 61-74.

Proctor, V.A. & Cunningham, F.E., 1988. The chemistry of lysozyme and its use as food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. 24, 359-395.

Ribéreau-Gayon, P., 1985. New developments in wine microbiology. Am. J. Enol. Vitic. 39, 1-10.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000a. The Microbiology of Wine and Vinifications. Handbook of Enology, vol. 1, John Wiley & Sons Ltd.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000b. The Chemistry of Wine and stabilization and Treatments. Handbook of Enology, vol. 2, John Wiley & Sons Ltd.

Rice-Evans, C.A., Miller, N.J. & Paganda, G., 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Rad. Res. 22, 375-383.

Rice-Evans, C.A., Miller, N.J. & Paganda, G., 1997. Antioxidant properties of phenolic compounds. Trend Plant Sci. Rev. 2, 152-159.

Ruiz, A., Poblet, M., Mas A. & Guillamon, J.M., 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int. J. Syst. Microbiol., 50, 1981-1987.

Stead, D., 1993. The effect of hydroxycinnamic acids on the growth of wine-spoilage lactic acid bacteria. J. Appl. Bact. 75, 135-141.

Stead, D., 1994. The effect of chlorogenic, gallic and quinic acids on the growth of spoilage strains of *Lactobacillus collinoides* and *Lactobacillus brevis*. Lett. App. Micobiol. 18, 112-114.

Thom, H.B., 1958. Journal of Jan Van Riebeeck, Volume III 1659-1662. A.A. Balkema, Cape Town, Amsterdam.

Usseglio-Tomasset, L., 1992. Properties and use of sulphur dioxide. Food Addit. Contam. 9, 399-404.

Uthurry, C.A., Suárez Lepe, J.A., Lombardero, J. & García Del Hierro, J.R., 2006. Ethyl carbamate production by selected yeasts and lactic acid bacteria in red wine. Food Chem. 94, 262-270.

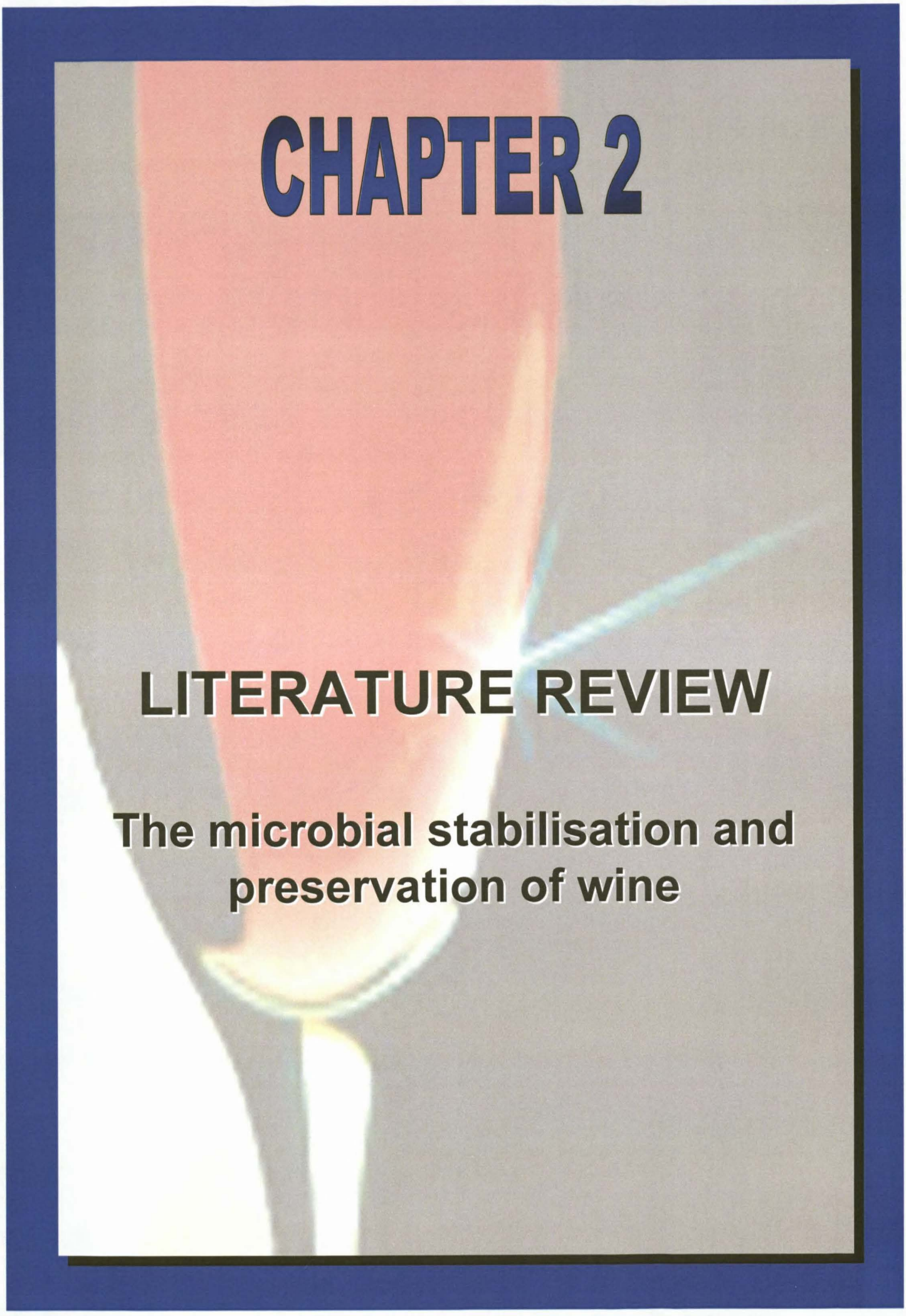
Winkler, A.J., Cook, J.A., Kliewer, W.M. & Lider, L.A., 1974. General viticulture. University of California Press, Berkeley.

Yamada, Y., Hoshino, K. & Ishikawa, T., 1997. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: The elevation of the subgenus *Gluconobacter* to the generic level. Biosci. Biotech. Biochem., 61, 1244-1251.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nurry, F.S., 1995. Wine Analysis and Production. Chapman & Hall, New York.

http://www.lysozyme.com

http://www.sawis.co.za



CHAPTER 2

LITERATURE REVIEW

The microbial stabilisation and preservation of wine

2. LITERATURE REVIEW

2.1 INTRODUCTION

Wine has been made since pre-recorded history. Biblical scrolls indicate that the Egyptians made and consumed wine several thousands of years ago. Also many a king or foe has written poems about the magical and mysterious powers of wine. Before the days of modern medicine, wine had many uses as preservative, disinfectant and intoxicating agent, naturally due the ethanol content. Buckenhüskes (1993) wrote that fermentation was not reserved to wine only, because the origin of fermented foods is thought to be from the Orient which dates back to prehistoric times. Initially, fermentation processes, e.g. alcoholic, acetic acid and lactic acid fermentation, were mainly used to preserve foods of animal and plant origin (Buckenhüskes, 1993).

Wine has been regarded as restorative, a stimulant, an appetiser, and even as an analgesic for many body aches, but also as an evil product when consumed in excess (Van de Wiel *et al.*, 2001). There is evidence that moderate wine consumption, especially red wine, have a positive effect on human health. This is due to phenols found in grape skins, seeds and vine stems. Phenols act as antioxidants, antimicrobials and modulators of various enzyme systems (Rice-Evans *et al.*, 1995, 1997; German & Walzem, 2000; De Beer *et al.*, 2002).

Today there is more emphasis on product management, competition from other producers and countries. Winemaking and maturation processes have intensively been studied by scientist with considerable progress over the past few years; therefore winemakers are now able to optimise processes to achieve the optimum wine quality. The wine industry in South Africa has developed into a multi-million Rand business, supplying work to thousands of people. A cellar or producer can suffer huge economical damages if wine is spoiled by microorganisms, oxidation or any other process that can be detrimental to wine quality.

The uncontrolled proliferation of microorganisms eventually leads to product deterioration and spoilage (Zoecklein *et al.*, 1995). Thus, oenologists are trying to control all stages of winemaking. Concerns regarding wine production include potential financial loss due to biotic contamination of wines, either by fungi from corks or by yeasts, lactic acid bacteria (LAB) and/or acetic acid bacteria (AAB) (Ubeda & Briones, 1999).

This literature study is therefore focused on the microorganisms associated with the winemaking process and methods to stabilise and preserve wine microbiologically. Special preference will be given to LAB and factors, negative as well as positive, that influence LAB growth and malolactic fermentation (MLF). Preservation methods that will be discussed are physical, chemical and biological, and lysozyme will be the major focus in the biological preservation section as the mechanism to prevent LAB growth and therefore spoilage in wine.

2.2 THE WINEMAKING PROCESS AND ASSOCIATED MICROORGANISMS

At harvest time grape berries contain a large diversity of microorganisms including yeasts, LAB, AAB and moulds. The yeast *Saccharomyces cerevisiae* and the LAB *Oenococcus oeni* [formerly *Leuconostoc oenos* (Dicks *et al.*, 1995)] have a positive contribution in winemaking. Other LAB genera found in wine are species of *Leuconostoc*, *Pediococcus* and *Lactobacillus* (Ribéreau-Gayon *et al.*, 2000a). Most lactobacilli and pediococci are considered undesirable or spoilage bacteria because of cosmetic and flavour depreciation of wine. Undesirable effects on wine include spoilage, off-flavours, colour changes and formation of biogenic amines (Edwards *et al.*, 1998a, b, 1999; Lonvaud-Funel, 1999; Maicas *et al.*, 1999; Du Toit & Pretorius, 2000; Guerrine *et al.*, 2001).

The control of yeast and bacteria growth is necessary in winemaking to ensure a high quality product. MLF follows the alcoholic fermentation (AF) and when completed, sulphur dioxide (SO_2) is adjusted to levels that will protect wine against spoilage and/or oxidation. Until recently, SO_2 was at this stage of winemaking the most efficient agent for the microbiological stabilisation of wine. Today, lysozyme can also be used as an effective agent to stabilise wine against LAB (Lonvaud-Funel, 1999; Farías & Manca de Nadra, 2000; Mira de Orduña *et al.*, 2000, 2001; Vidal *et al.*, 2001; Saguir & Manca de Nadra, 2002).

Several authors described that MLF is the bioconversion of the dicarboxylic malic acid in wine to the monocarboxylic lactic acid and carbon dioxide (CO_2) (**Fig. 2.1**). Besides de-acidifying the wine, MLF improves the biological stability of wines by preventing malic acid utilisation by other non-desirable LAB species. LAB can also affect the final aroma balance of wine by modifying fruity aromas and have the potential to produce aroma-active compounds such as diacetyl. The induction of MLF in wines with selected LAB strains can offer a positive contribution to the final aroma in wines. Descriptive terms for flavour enhancement include buttery, nutty, yeasty, oaky and sweaty aromas. Therefore, different strains of LAB could have different sensory effects on wines (Edwards *et al.*, 1991 ; Strasser de Saad & Manca de Nadra, 1992 ; Buckenhüskes 1993; Nielsen *et al.*, 1996 ; Sauvageot & Vivier, 1997 ; Maicas *et al.*, 1999 ; Farías & Manca de Nadra, 2000 ; Grimaldi *et al.,* 2000; Mira de Orduña *et al.*, 2000 , 2001 ; Vidal *et al.*, 2001).

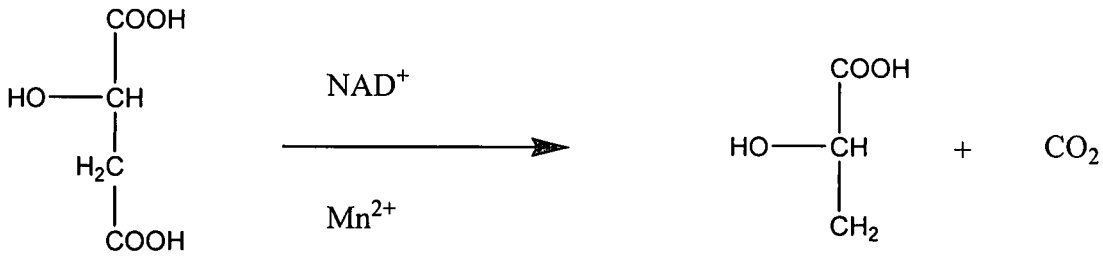


Figure 2.1 The basic process of MLF by LAB (Boulton *et al.*, 1996). The malolactic enzyme is different from the malic enzyme leading to pyruvate. Because of the optimum pH of the enzyme (around 5.8) the need for cofactors (Mn^{2+}, NAD^+) are necessary (Lonvaud-Funel, 1999).

2.2.1 YEASTS

Wine yeasts are responsible for the primary AF when sugar (mainly glucose and fructose) are converted by a unique metabolic pathway, known as glycolysis (**Fig. 2.2**) (Walker, 1998), to mainly ethanol and CO_2.

$$2\ C_6H_{12}O_6 + 2\ Pi + 2\ ADP \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP + 2H_2O$$

Figure 2.2 Simplified figure of glycolysis (Walker, 1998).

Several yeast genera en species are present on grapes, in winemaking and cellar equipment and have adapted to various conditions and environmental stresses to survive these harsh conditions. These yeasts include non-*Saccharomyces* genera such as *Brettanomyces*, and its anamorph *Dekkera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulopsis*, *Torulaspora*, and *Zygosaccharomyces* (Ribéreau-Gayon, 1985; Boulton *et al.*, 1996; Fleet, 1998; Pretorius *et al.*, 1999; Zoecklein *et al.*, 1995) These non-*Saccharomyces* yeasts are mainly associated with wine spoilage and have been implicated in several cases of turbidity and film formation as well as the production of off-flavours (Du Toit & Pretorius, 2000). The wine yeast *S. cerevisiae* is the dominant yeast at the end of AF. Today it is general practise to inoculate must with commercial starter cultures of *S. cerevisiae* at 10^6 colony forming units per (CFU) per millilitre (mL). This procedure is done to complement the naturally low numbers of *S. cerevisiae* at the beginning of AF, thus ensuring that the dominant yeast is *S. cerevisiae*. The advantages of inoculating with a commercial starter culture of *S. cerevisiae* include faster onset and rate of AF, higher alcohol tolerance by the yeast and improved sensory quality of the wine (Walker, 1998).

Many factors influence the AF and these include sugar content (mainly glucose as carbon source), oxygen (O_2) (for yeast growth), assimilable nitrogen availability such as ammonium (NH_4^+) or amino acids, phosphate, mineral elements, growth factors (vitamins, purines and pyrimidines, nucleosides and nucleotides, 'survival factors' or long chain fatty

acids and sterols), killertoxins, SO_2 and pesticide residues (Walker, 1998; Lourens & Reid, 2002).

2.2.2 LACTIC ACID BACTERIA

LAB are Gram-positive, catalase-negative, non-motile, non-sporeforming, rod- and coccus shaped. LAB are divided into three groups according to their metabolic activity: obligatory homofermentative, facultative heterofermentative and obligatory heterofermentative. Homofermentative LAB reduces hexose sugars to lactic acid via the Embden-Meyerhof-Parnas (glycolytic) pathway where heterofermentative lactobacilli, leuconostocs and oenococci produce D-lactic acid and acetic acid through the 6-phosphogluconate pathway (Du Toit & Pretorius, 2000) (**Fig. 2.3**).

The activity of heterofermentative LAB in wines causes a marked increase in volatile acidity (VA) and acetic acid was correlated with fructose utilisation. Lactobacilli belong to facultative (*Lactobacillus plantarum, L. casei)* and obligatory (*L. hilgardii, L. brevis, L. fructivorans*) heterofermentative species. The homofermentative cocci are mainly *Pediococcus damnosus* and *P. pentosaceus*. Heterofermentative cocco-bacilli of wines are *Leuconostoc mesenteroides* and *O. oeni* (Strasser de Saad & Manca de Nadra, 1992; Buckenhüskes 1993; Caplice & Fitzgerald, 1999; Du Toit & Pretorius, 2000; Mira de Orduña *et al.*, 2001).

Apart from glucose fermentation, LAB of wine can convert malic acid to lactic acid (MLF) via a unique energy-producing [ATP via the membrane bound ATPase (Henick-Kling *et al.*, 1998)] pathway. This pathway includes an energy gradient producing transport of malic acid into the cell, the intracellular decarboxylation via the malolactic enzyme, and the efflux of lactic acid possibly with one hydrogen ion (Henick-Kling *et al.*, 1998).

Some heterofermentative LAB can also degrade L-arginine, one of the most important amino acids in grape must and wines via the arginine deiminase (ADI) pathway leading to the production of ammonia, ornithine, ATP and CO_2. Liu & Pilone (1998) proved that the arginase-urease pathway implicated urea formation. Citrulline is an intermediate in the ADI pathway and is excreted during the degradation of arginine. The production of ammonia increases the pH and the risk of growth of spoilage microorganisms. Cittruline is a precursor in the formation of carcinogenic ethyl carbamate (EC) (urethane). The formation of EC is a spontaneous chemical reaction involving ethanol and a compound that contains a carbamyl group such as urea, citrulline and carbamyl phosphate. Several authors have reported a linear proportionality between arginine degradation and citrulline degradation in studies with resting cells of LAB. Canada has a legal EC limit of 30 µg/L and in the United States of America (USA) there is a voluntary limit of 15 µg/L (Arena *et al.*, 1999, 2002; Vergès *et al.*, 1999; Mira de Orduña *et al.*, 2000, 2001).

Mira de Orduña *et al.* (2001) advised to use non-arginine-degrading pure oenococcal cultures of *O. oeni* for the induction of MLF. One can thus conclude that arginine

degradation and citrulline reutilisation are different for different genera, species and strains of LAB.

LAB are present on grapes or inoculated into wine, hence Edwards *et al.* (1991) studied the isolation and characterisation of native strains of *O. oeni* and found that native strains differed in their sensitivity towards temperature and SO_2. They also found that native strains induced a faster MLF than commercial strains (Edwards *et al.*, 1991). Edwards & Jensen's (1992) studies continued with the occurrence and characterisation of *Pediococcus* spp. from Washington State wines.

Edwards *et al.* (1993) continued with important research on *Lactobacillus* spp. from Washington State wines. They proved that not all species of *Lactobacillus* are involved in

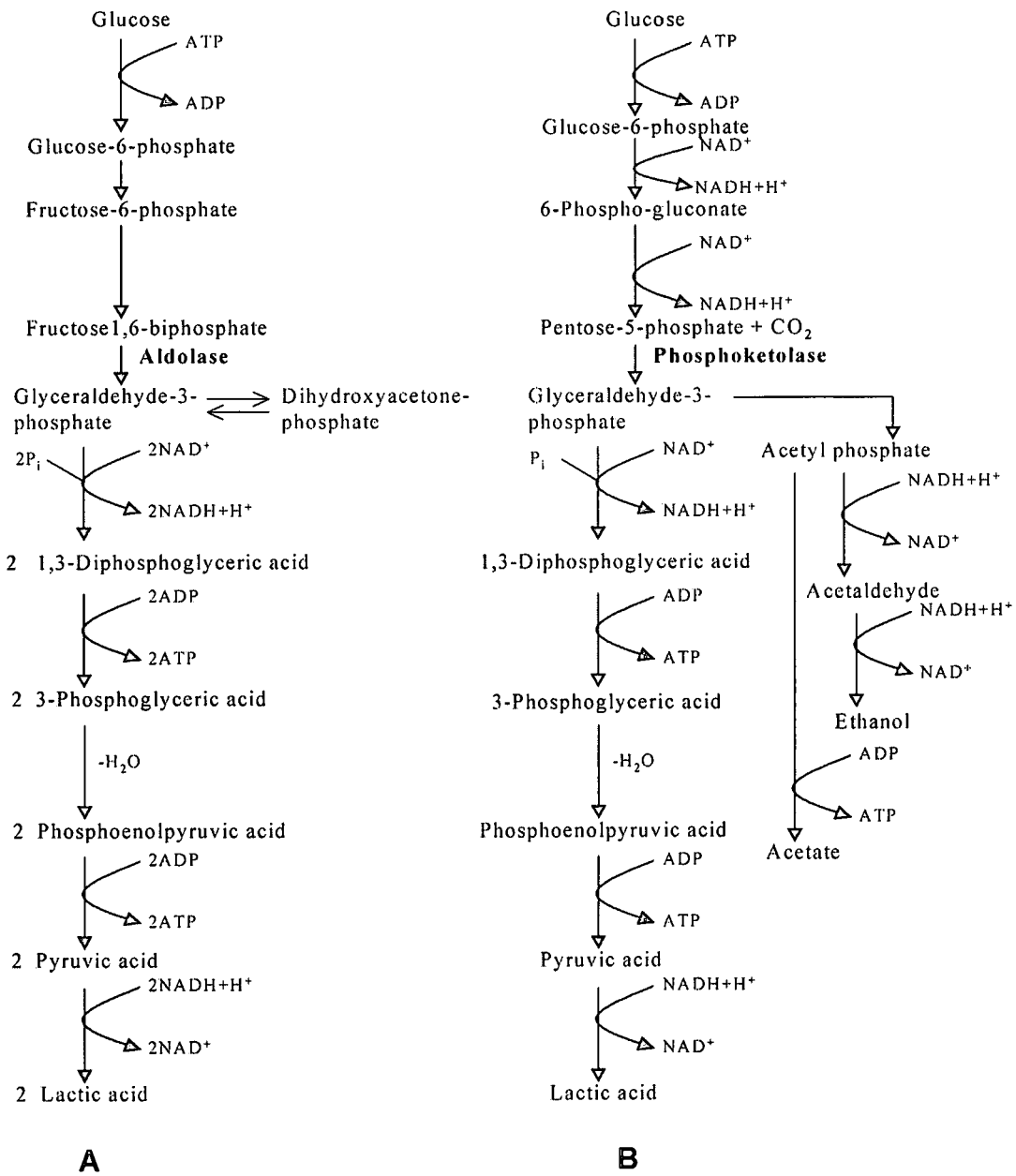


Figure 2.3 (A) Embden-Meyerhof-Parnas pathway (glycolysis) of homofermentative LAB and (B) 6-phosphogluconate pathway of heterofermentative LAB (Du Toit & Pretorius, 2000).

stuck AF and that strains had different tolerances to low pH and high concentrations of SO_2 and ethanol. Five years later Edwards *et al.* (1998a) proposed a new species of *Lactobacillus*, namely *Lactobacillus kunkeei*. *L. kunkeei* has since been labelled as the "ferocious lactobacilli", able to induce stuck and/or sluggish AF due to the production of high amounts of acetic acid. In most cases *O. oeni* dominates at the end and after AF.

Several authors have reported about the stress conditions and subsequently the adaptation of LAB in wine to conditions such as heat shock (low temperature), ethanol, SO_2, fatty acids, low pH and low amino acid concentration (Edwards & Jensen, 1992; Edwards *et al.*, 1993, 1998a, 1998b, 1999, 2000; Du Toit & Pretorius, 2000; Carreté *et al.*, 2002). Farías & Manca de Nadra (2000) found that starved cells of *O. oeni* produces exoprotease, suggesting that it could be a mechanism for survival in the stress conditions encountered in winemaking. Carreté *et al.* (2002) explained that the survival of *O. oeni* is related to the structure and function of the plasma membrane and its compounds. If the plasma membrane is altered, it will lose its semi-permeability and enzymatic properties thus resulting in cell death. ATPase is bound to the plasma membrane in LAB. Its function is essential for growth since the ATPase proton pump, coupled with ATP hydrolysis, extrudes protons from the cell to the media, thus controlling the intracellular pH. The activity of ATPase depends on proton transport demand and substrate catabolism. Since the pH of wine is very low, ATPase activity is very important (Carreté *et al.*, 2002).

Several authors studied freeze dried cultures of LAB for direct inoculation into wine to ensure that the correct species conducts the MLF. LAB are generally weak proteolytic and lypolytic and require preformed amino acids, purine and pirimidine bases and B-vitamins for growth. Spontaneous MLF occurs by indigenous flora of LAB, originating from the vines, grape skins and winery equipment. These spontaneous MLFs may take several months and are quite unpredictable, not knowing which genus or species are the dominant flora. Commercial cultures of *O. oeni* ensure better control on the time of onset and the rate of MLF, reduce the potential of spoilage by other bacteria, and reduce potential interference by bacteriophages. Nielsen *et al.* (1996) demonstrated the 100% survival of a freeze-dried preparation of a selected *O. oeni* strain into wine. The winemaker can now pay more attention to the control of the flavour modifications induced by *O. oeni*. There are several selection criteria for LAB for the induction MLF in wine (Buckenhüskes, 1993; Nielsen *et al.*, 1996; Sauvageot & Vivier, 1997; Nielsen & Richelieu, 1998; Caplice & Fitzgerald, 1999; Maicas *et al.*, 1999; Farías & Manca de Nadra, 2000; Mira de Orduña *et al.*, 2000, 2001).

2.2.3 ACETIC ACID BACTERIA

Acetic acid bacteria are Gram-negative, catalase positive rods (De Ley *et al.*, 1984; Holt *et al.*, 1994). In wine, AAB are associated with wine spoilage. The species associated with grapes and wine are of the genera *Acetobacter* (*A. aceti*, and *A. pasteurianus*), *Gluconobacter* (*G. oxydans*) (Joyeux *et al.*, 1984a, b; Drysdale & Fleet, 1988) and

Gluconoacetobacter (*Gl. liquefaciens and Gl. hansenii*) (Yamada *et al.*, 1997; Ruiz *et al.*, 2000). One of the most common differences between *Gluconobacter* and *Acetobacter* is the ability of *Acetobacter* to oxidise ethanol to acetic acid and the latter into CO_2 and water (H_2O) (Du Toit, 2000). *Gluconobacter* can only oxidise ethanol to acetic acid.

The increase in acetic acid causes the VA to increase. The increase in VA is undesirable from a sensorial perspective and depending on the type of wine produced, usually has a distinctive acetic acid, nutty, green apple or paint like aroma. The production of acetic acid may also contribute to sluggish or stuck AF (Du Toit & Pretorius, 2002). *Gluconobacter* dominates at the start of fermentation when the main carbon source is glucose, thus indicating their preference for a sugar-rich environment. Normally their numbers decrease to between 0 and 10^2 cells per mL at the end of AF (Joyeux *et al.*, 1984a, b; Drysdale & Fleet, 1989; Du Toit & Lambrechts, 2002). Du Toit & Lambrechts (2002) found that the majority of *Acetobacter* species occurring at the end of AF were *A. pasteurianus* and *A. liquefaciens* [now classified as *Gl. liquefaciens* (Du Toit & Pretorius, 2002)].

Many countries have imposed a strict limit on the maximum VA concentration in wines. The legal limit for VA in South Africa is 1.2 g/L, but can be detrimental to quality from 0.8 g/L and up (http://www.sawis.co.za). New legislation includes lowering VA levels by a reverse osmosis technique, but is relatively expensive. However, research has shown that treated wines were watery, flat and lacked character. It would be advisable to blend these treated wines with untreated wines (Lambrechts, personal communication).

The numbers of AAB increases dramatically on rotten or *Botrytis* infected grapes, with cell counts as high as 10^6 cells per mL being recorded. When this happens, *Acetobacter* species start to dominate. This may be due to the ethanol production by wild yeast occurring on the damaged grapes (Joyeux *et al.*, 1984a, b). *Acetobacter* numbers can increase in the middle and later stages of AF due to the increase in ethanol concentration, thus indicating that *Acetobacter* species prefer ethanol as a carbon source (De Ley *et al.*, 1984; Joyeux *et al.*, 1984a, b; Drysdale & Fleet, 1985). The pumping over or racking of wines may lead to the uptake of small amounts of O_2. This may lead to the survival and potential growth of AAB in wine.

Spoilage of bottled wines can possibly be controlled by sufficient levels of SO_2, lack of O_2 and the use of sterile filtration techniques at the time of bottling. Some authors stated that storing bottled wine in a horisontal position reduces the accelerated oxidative spoilage of wine, having in mind that good bottle closures are used (Bartowsky *et al.*, 2003). Factors influencing the growth of AAB in must and wine include ethanol, pH, acidity, O_2, SO_2 and temperature (Joyeux *et al.*, 1984a; Drysdale & Fleet, 1985; Du Toit, 2000). However, Du Toit & Lambrechts (2002) pointed out that sensitivity of AAB to pH, SO_2 and ethanol is strain dependant and that it is advisable to start an AF with a low pH (< 3.6) and sufficient levels (e.g. 50 mg/L) of SO_2.

2.3 FACTORS INFLUENCING THE GROWTH OF AND MALOLACTIC FERMENTATION BY LACTIC ACID BACTERIA

2.3.1 pH

According to Lonvaud-Funel (1999) the growth of LAB is mainly correlated with pH, with higher pH levels leading to increased LAB populations. Davis *et al.* (1986) stated that pH has a profound and selective effect upon LAB species that grow in wine. They also showed that the growth rate of *O. oeni* increased as the wine pH increases from 3.2-4.0, and MLF occurred in conjunction with growth. Wibowo *et al.* (1988) confirmed that the rate of MLF, conducted by *O. oeni*, increased as wine pH increased from 3.1 to 3.8. Liu & Gallander (1983) indicated that the highest rate of MLF occurred at high (> 3.5) initial pH and low SO_2 levels. Davis *et al.* (1986, 1988) illustrated that *O. oeni* had a greater tolerance to low pH values and that explains the almost exclusive isolation of this species from wine with a pH below 3.5. High pH (>3.5) wines usually contain species of the genera *Lactobacillus* and *Pediococcus* (Davis *et al.*, 1986; Du Toit & Pretorius, 2000).

In a study Britz & Tracey (1990) found that a decrease in pH had a negative affect on the growth of *O. oeni* and this effect was enhanced by lower temperatures. They also concluded that lower pH and higher SO_2 concentrations had the greatest inhibitory effect on the growth of *O. oeni* (Britz & Tracey, 1990). Salou *et al.* (1991) indicated that the optimal pH for *O. oeni* growth on complex media is 4.5, thus indicating that the growth medium also plays a role when determining LAB cell counts in wine.

The pH also affects the malolactic activity of the bacterial cell. Hence, the MLF rate depends not only on the activity but also the quantity of cells present in wine. The pH is therefore very important and influences several aspects regarding LAB in wine i) in the selection of the most adaptable strains; ii) in the growth rate and yield; iii) the malolactic activity of the specific species of LAB; and iv) even in the nature of the substrates transformed (Boulton *et al.*, 1996; Ribéreau-Gayon *et al.*, 2000a).

2.3.2 ETHANOL

Britz & Tracey (1990) reported that ethanol is generally regarded as the principle inhibitor of bacterial growth in wine. The resistance to ethanol by bacteria varies from strain to strain and is influenced by other conditions in the medium. Carreté *et al.* (2002) explained that ethanol interacts with cell membranes by becoming part of their hydrophobic structure. This increases the polarity of the region and affects hydrophobic interactions. Moreover, it can affect the positioning of proteins within the membranes and therefore the ATPase function of LAB (Carreté *et al.*, 2002).

In the study of Guerzoni *et al.* (1995) oenococcis growth rate attained a maximum at ethanol concentrations of five to six percent and decreased again at higher levels. Wibowo *et al.* (1988) found a seven day delay of MLF when the ethanol concentration was increased from 10% to 13%. Capucho & San Romão (1994) observed no *O. oeni* growth

at an ethanol concentration of 14% although 70% of the malic acid was degraded. Ribéreau-Gayon *et al.* (2000a) described that *O. oeni* is inhibited in environments rich in ethanol and struggle to grow at or above 13-14 percent volume. The ethanol tolerance of laboratory strains is much less than for the same strains cultivated in wine. Hence, Lonvaud-Funel (1999) found that the natural selection of *O. oeni* during AF is mainly due to the progressive increment in ethanol and other products of yeast metabolism. Ethanol is known to alter the bacterial membrane (Lonvaud-Funel, 1999).

Davis *et al.* (1988) found that certain species of *Lactobacillus* were more tolerant to higher ethanol concentrations than *Pediococcus* spp. or *O. oeni.* Stratiotis & Dicks (2002) found species of *L. vermiforme*, *L. casei* subsp. *casei*, *L. buchneri* and *L. plantarum* in spoiled fortified wine at 22% (vol/vol) ethanol. Edwards & Jensen (1992) reported that pediococci possessed different tolerances to ethanol. They also concluded that the native strains of *Pediococcus* have a weak or incapacity to catalyse MLF in wines.

2.3.3 TEMPERATURE

Temperature influences the growth rate of all microorganisms. It accelerates biochemical reactions and consequently growth varies with temperature according to a bell curve. Several authors speculated that the optimum growth temperature for *O. oeni* is from 27 to 30°C, but this is not the same in an alcoholic matrix e.g. wine. The optimum temperature range for *O. oeni* growth in wine varies between 20-25°C. Temperatures of 15°C and lower decrease the possibility of oenococcal growth (Beelman *et al.*, 1977; Britz & Tracey, 1990; Ribéreau-Gayon *et al.*, 2000a). Edwards *et al.* (1999) found that *O. oeni* YH-37's exhibited growth at temperatures ranging between 21-32°C, thus indicating a difference between strains. Different parameters should also be taken into account when optimum growth temperatures are investigated.

Britz & Tracey (1990) proved that a decrease in temperature (from 25°C to 15°C) caused a significant effect on the growth of the tested *O. oeni* strains.

The ideal temperature for LAB, especially *O. oeni,* and for malic acid degradation in wine is around 20°C. The onset of MLF is delayed and longer time is needed for MLF completion when temperatures are higher or lower than 18°C (Ribéreau-Gayon *et al.*, 2000a).

2.3.4 SULPHUR DIOXIDE

When all malic acid is degraded, wines are stabilised by sulphating. Most of the bacteria and possibly the remaining yeasts are sensitive to SO_2. In high pH wines, the ineffectiveness of SO_2 (lower molecular SO_2 levels) of the medium mean that bacteria survive more easily (Lonvaud-Funel, 1999). Britz & Tracey (1990) found that low pH values and high SO_2 concentrations had the greatest inhibitory effect on *O. oeni.* This is due to a greater proportion of the undissociated, antimicrobial free form of SO_2 (Davis *et al.*, 1986). Davis *et al.* (1988) confirmed that *O. oeni* to be the least tolerant of higher

SO_2 concentrations. Guzzo *et al.* (1998) confirmed these findings by indicating that 60 mg/L of SO_2 (pH 3.5) resulted in total death within 24 hours of *O. oeni*. Interestingly, they also found that by growing *O. oeni* in an acidic medium (pH 3.5) without SO_2 led to a two log higher cell count in comparison to non-adapted cells when incubated in a medium containing 30 mg/L of SO_2, indicating SO_2 tolerance in response to acidic treatment.

Total SO_2 in wine is the sum of its free and bound forms. Its effectiveness as an antimicrobial agent and as an antioxidant is directly linked to wine composition and pH. SO_2 penetrates into the cell in the molecular form by diffusion. In the cytoplasm where the pH is lowest, it dissociates and reacts with essential biological molecules such as enzymes with disulfide bonds, coenzymes and vitamins. This results in cessation of growth and finally cell death (Ribéreau-Gayon *et al.*, 2000a; Carreté *et al.*, 2002).

Wines of high total SO_2 concentration are more likely to undergo natural MLF which can be attributed to species of *Pediococcus* or *Lactobacillus*. This may be undesirable for sensory quality (Davis *et al.*, 1988). Liu & Gallander (1983) found that the largest decrease in bacterial population was observed at pH 3.3 and at 75 ppm of SO_2. Edwards *et al.* (1999) found a good correlation between lowering pH values (< 3.5) prior to fermentation and the antimicrobial action of molecular SO_2, subsequently resulting in effective control of the growth of *L. kunkeei* (Edwards *et al.*, 1999).

Osborne *et al.* (2000) reported on the inhibitory effect of acetaldehyde-bound SO_2 against LAB. They suggested that LAB metabolises the acetaldehyde fraction, yielding free SO_2 and thus inhibiting LAB growth. Thus, depending on the wine style, it may be beneficial to use efficient acetaldehyde-degrading strains in white wines with high acetaldehyde concentrations after the AF. The degradation of SO_2-bound acetaldehyde by SO_2-sensitive LAB strains may therefore play a role in causing stuck or sluggish MLF (Osborne *et al.*, 2000).

Carreté *et al.* (2002) found that the greatest inhibition of *O. oeni* was in the presence of SO_2 and at 42°C, with the MLF taking up to 40 days for a wine containing malic acid. Cell growth was delayed and decreased and in the presence of SO_2 the specific ATPase activity was inhibited (Carreté *et al.*, 2002). Wibowo *et al.* (1988) showed a four week delay in completion of MLF when the total SO_2 concentration increased from 0 to 43 mg/L.

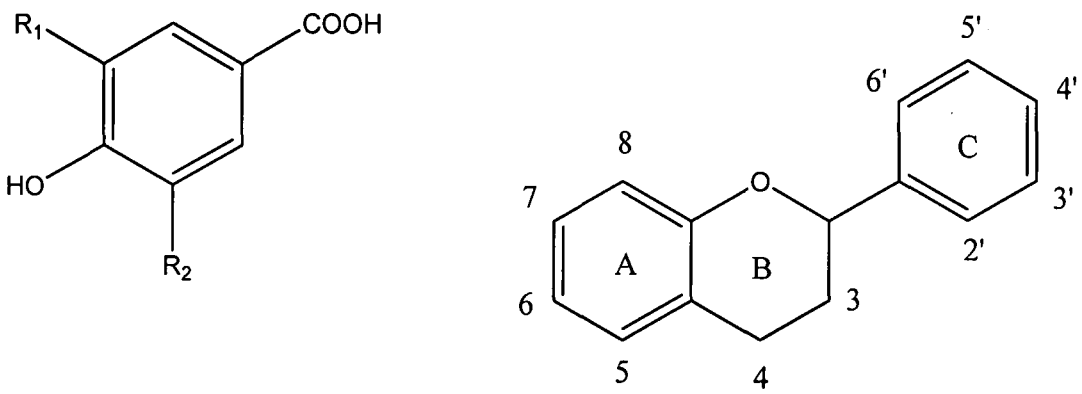
The inhibitory action of SO_2 on the malolactic enzyme of *Oenococcus* is in addition to its effect on cellular growth. As a general rule, LAB have difficulty in growing at concentrations exceeding 100 mg/L total SO_2 and 10 mg/L free SO_2. Strains vary in their SO_2-sensitivity and this can be related to environmental growth conditions and physiological adaptation possibilities (Ribéreau-Gayon *et al.*, 2000a).

2.3.5 PHENOLIC COMPOUNDS

The great number of phenolic compounds in red wine could be one of the main causes of MLF inhibition in red wine. The amounts of phenolic compounds contained in red wines essentially depend on the grape variety, vinification process and barrel ageing: phenol

carboxylic acids (PCA), 100-200 mg/L, catechin, 10-400 mg/L; and anthocyanins, 20-500 mg/L, with condensed tannins (1-3 g/L) and oak tannins also occuring. Some PCA can inhibit the growth of LAB and others can stimulate MLF carried out by *O. oeni*. Phenolic acids appear mainly in a combined form: hydroxycinnamic acids form esters with tartaric acid (cynammoyl-tartaric acids) and hydroxybenzoic acids polymerise with other molecules to produce wood tannins. Campos *et al.* (2003) stated that the inhibitory effect of hydroxycinnamic acids is stronger than hydroxybenzoic acids, and that caffeic and ρ-coumaric acid were the most inhibitory compounds when tested against *O. oeni*. Some lactobacilli can metabolise hydroxycinnamic acids, producing 2-hydroxyphenylpropionic acids which can, in turn, be decarboxylated yielding substituted ρ-ethyl phenols. These compounds are often described as off-flavours in wine with low taste thresholds (Vivas *et al.*, 1997, 2000 ; Reguant *et al.*, 2000; Campos *et al.*, 2003; Rozès *et al.*, 2003).

Some phenolic compounds such as gallic acid (trihydroxybenzoic acid) and monoglucoside anthocyanins seem to have a positive effect on LAB growth and MLF. Stead (1993) demonstrated that free hydroxycinnamic acids affected the growth of spoilage LAB in laboratory media as they are stimulatory at low concentrations but inhibitory at high concentrations. Stead (1994) reported that gallic acid, chlorogenic acid and quinic acid stimulated growth during the early stages of growth of *L. collinoides*, but not for *L. brevis*. Hence, these acids are more likely to increase, rather than decrease the risk of spoilage from LAB (Stead, 1994). It is suggested that LAB use the glucose moiety of the anthocyanin as an energy source (Reguant *et al.*, 2000; Vivas *et al.*, 1997, 2000; Campos *et al.*, 2003; Rozès *et al.*, 2003).



A **B**

Figure 2.4 Examples of basic structures of phenolic compounds commonly found in wine. A: hydroxybenzoic acid and B: basic structure of a flavanol (De Beer *et al.*, 2002).

Procyanidins in grapes and ellagitannins in oak wood seem to have a strong inhibiting effect on LAB growth and MLF by several modes of action: they can inhibit enzyme activity, adhere to cell walls or form complexes with copper and iron. Phenolic compounds may also damage the bacterial cell membrane, generally causing leakage of intracellular constituents. Gram-positive bacterial polysaccharides, such as peptidoglycan in *O. oeni*,

allow hydrogen links to form between the cell walls of bacteria and tannins. In contrast, the cell wall structure of Gram-negative bacteria, in particular the presence of the external lipid layer protects the cells from the inhibiting effects of tannins (Reguant *et al.*, 2000; Vivas *et al.*, 2000; Campos *et al.*, 2003; Rozès *et al.*, 2003).

Pure ellagitannins in the form of vescalagin improved the overall viability of the bacterial population, unlike wood extract, which proved to be toxic. The beneficial effect of vescalagin on the bacterial viability may be caused by the β-glucosidase activity of *O. oeni*, which allows the cells to utilise the glucose in the ellagitannin. Procyanidins are inhibitors only in the native form. Their inhibiting effect decreased with their degree of oxidation (Vivas *et al.*, 2000).

One can thus predict that the onset of MLF will be problematic in wines, which are rich in oligomeric procyanidins. This situation particularly arises when grapes are harvested before reaching optimum sugar levels, when seeds are still too rich in tannins (Vivas *et al.*, 2000).

Rozès *et al.* (2003) reported that the presence of phenolic compounds induced a shift from the consumption of glucose and fructose, thus reducing the rate of sugar consumption. Phenolic compounds also seem to play a role in citric acid consumption as they do in malic acid degradation (Rozès *et al.*, 2003).

2.3.6 PESTICIDES

Chemical pesticides, mainly fungicides, are used to fight harmful diseases in viticulture such as powdery mildew, downy mildew and *Botrytis cinerea*. Some of these diseases develop in the last phase of grape ripening, and growers sometimes have to apply chemical treatment just before harvesting, which can lead to pesticide residue in grapes. Legal maxims have been established in various countries and minimal safety periods are obligatory between the final applications of pesticides and harvesting (**Table 2.1**). These periods are not always strictly respected, and pesticide residues can be found in musts and wines. These residues have the potential to affect yeasts and may cause sluggish or stuck AF. Pesticide residues can also affect LAB in wine and delay the MLF (Garcia-Cazorla & Xirau-Vayreda, 1994; Vidal *et al.*, 2001).

Garcia-Cazorla & Xirau-Vayreda (1994) found that Iprodine and Vinclozin (dicarboximidic fungicides) levels decreased during winemaking. Vidal *et al.* (2001) found that copper and dichlofluanid's minimal inhibitory concentrations that affected MLF are just under 5 mg/L, which is enhanced by ethanol, low pH and SO_2. Inhibition is due mainly to a decrease in cell number but not to lower specific malolactic activity (Vidal *et al.*, 2001).

Carreté *et al.* (2002) found that 20 mg/L copper reduced specific ATPase activity of *O. oeni*. The inhibitory effect of copper may be due to competition with Mg^{2+} for ATPase (Carreté *et al.*, 2002). The sensitivity of several strains of LAB to the pesticides varies, even within the same species (Vidal *et al.*, 2001).

It is, however, possible to remove or lower the potential pesticide residues from wine by the means of filtration and filter agents. The study of Ruediger *et al.* (2004) indicated that bentonite and activated carbon was the most effective in lowering/absortion of the tested pesticides commonly used in Australia.

Table 2.1 Pesticides, their active compounds, maximum concentration found in grapes and the maximum concentration found in wines (Vidal *et al.*, 2001).

Pesticide	Active compound	Legal max. concentration in crushed grapes (mg/kg)	Max. concentration found in wine (mg/L)
$CuSO_4 \cdot 5H_2O$	Cu^{2+}	20	5
$CuCl_2 \cdot 2H_2O$	Cu^{2+}	20	5
Copper oxychloride	Cu^{2+}	20	5
Euparen	Dichlofluanid	10	5
Dubarasan	Chloropyrifos	0.5	< 0.1
Metilparafene	Parathion-methyl	0.2	< 0.1
Sumithion	Fenitrotion	0.5	0.1
Rovral	Iprodine	10	5
Ronilan	Vinclozolin	5	5
Sumiboto	Procymidone	5	2

2.3.7 FATTY ACIDS

Lafon-Lafourcade *et al.* (1984) reported that yeasts can produce compounds toxic to them which, if present in high concentrations, can result in a stuck AF. Decanoic (C6) and dodecanoic (C12) acids are the most common fatty acids present in wine (Capuco & San Romão, 1994) and levels vary from 0.64 to 14.0 mg/L depending on the cultivar and winemaking techniques (Lafon-Lafourcade *et al.*, 1984; Edwards & Beelman, 1987).

King & Beelman (1986) studied the inhibition of *O. oeni* growth by yeasts. They hypothesised that some metabolites other than ethanol and acetaldehyde produced by yeast may inhibit the bacteria. Lonvaud-Funel *et al.* (1988) showed that hexanoic-, octanoic- and decanoic acids were more inhibitory towards a *O. oeni* strain when added in combination than alone. Capuco & San Romão (1994) observed that decanoic acid, in concentrations up to 12.5 mg/L, and, dodecanoic acid up to 2.5 mg/L acted as growth factors stimulating also malolactic activity. At higher concentrations they exerted an inhibitory effect towards cell growth.

Caretté *et al.* (2002) found that dodecanoic acid inhibited the ATPase activity of *O. oeni* and that dodecanoic acid had a synergistic effect on ATPase with either high ethanol or low pH. Their studies pointed out that the toxicity of decanoic acid (C6) on LAB cells were significant in the presence of ethanol, probably because both compounds affect the cell membrane. Edwards & Beelman (1987) succeeded to facilitate a more rapid and predictable MLF by the use of yeast ghosts (hulls) to remove medium chain fatty acids (C6-C12).

Other authors found that the toxicity of octanoic acid increased when the pH of the medium decreased from 5.4 to 3.0 (Caretté *et al.*, 2002). The same result was observed for decanoic acid when Capuco & San Romão (1994) found that a decrease in pH from 6.0 to 3.0 clearly affects the malolactic activity of the tested *O. oeni* strain.

The undissociated form of fatty acid diffuses passively across the plasma membrane and is soluble in the hydrophobic portion. Thus a fraction of these fatty acids may be incorporated into the plasma membrane and modify its composition and permeability. The fatty acid can also enter the cell as protonated molecules and dissociate in the cytoplasm due the higher internal pH, leading to an increased intracellular hydrogenous concentration (decrease in intracellular pH and dissipation of the transmembrane proton gradient) (Capuco & San Romão, 1994; Caretté *et al.*, 2002).

Guerrini *et al.* (2001) investigated the influence of oleic acid on *O. oeni* and found that a few milligrams of oleic acid added to wine is sufficient to increase the high cell viability of LAB starter cultures and thus improving the chances of a more successful MLF.

2.4 CHEMICAL PRESERVATION

Combinations of preservation treatments allow the required level of protection to be achieved while at the same time retaining the organoleptic qualities of the product such as colour, flavour, texture and nutritional value (Brul & Coote, 1999). Preservatives also ensures microbiological stability and their maximum permitted concentration is strictly regulated by legislation. However, countries differ in their maximum levels of preservatives found and allowed in wine, therefore, it would be of high importance to know these levels and apply it in winemaking if planning to export. There is continued pressure from consumers for levels of chemical preservatives to be reduced (Stead, 1993). An ideal solution for the problem of decontamination, or disinfection, could be solved by the addition of compounds that when added quickly, inhibits indigenous microorganisms and also quickly degrades within 12-24 hours, without leaving toxic residues (Delfini *et al.*, 2002). However, these compounds have to have GRAS (Generally Regarded As Safe) status for human consumption.

2.4.1 PROPERTIES AND USE OF SULPHUR DIOXIDE

Sulphur dioxide's properties include an antimicrobial function that inhibits the development of microorganisms, and an anti-oxidative function that prevents oxidation of wine and/or musts. Results from literature showed that wine-related bacteria are more sensitive towards SO_2 than yeasts. Correct usage of SO_2 decreases the potential haze formation by yeasts, the re-fermentation of sweet wines, the development of flor yeasts and various bacterial defaults. Spoilage species of LAB and AAB as well as spoilage yeast genera such as *Brettanomyces* can be controlled effectively by the correct additions of SO_2. However, AAB vary in their sensitivity towards SO_2. *Acetobacter aceti* and *A. pasteurianus*

were found in wines with more than 50 mg/L total sulphites. AAB also grows in viable, but non-culturable state in wines containing SO_2 and up to 1.2 mg/L molecular SO_2 seems necessary to completely inhibit certain strains (Du Toit *et al.*, 2005).

SO_2 destroys yeast populations by inhibiting cellular multifunctions. Molecular SO_2 penetrates the cell by either active or passive transport or by simple diffusion. Once inside the cell it reacts with numerous constituents such as coenzymes, cofactors and vitamins influencing enzymatic reactions and nuleic acids, resulting in a decrease in ATP. SO_2 is also more effective on LAB than on yeasts. Originally SO_2 was obtained by burning sulphur (Stead 1993; Zoecklein *et al.*, 1995; Ribéreau- Gayon *et al.*, 2000a, b). Currently, the legal forms of SO_2 in South Africa are ammonium bisulphite, potassium- and sodium metabisulphite and compressed SO_2 gas. The legal maxim levels permitted for wines in South Africa differ according to the wine style from 160 mg/L total SO_2 (dry table wines), 300 mg/L total SO_2 (noble late harvests) and maximum 60 mg/L free SO_2 (http://www.sawis.co.za).

Juices and wines contain many readily oxidisable compounds, including polyphenols. In this regard, SO_2 serves as an anti-oxidant in must and wine. The browning phenomenon of fruit and grapes is the result of the activity of a group of plant enzymes, the tyrosinases (polyphenoloxidase). These enzymes catalyse oxidation of non-flavonoid o-dihydroxy phenols (colourless) to their corresponding darkened quinines. Laccase, unlike tyrosinases, are present on grapes infected and degraded with *Botrytis cinerea.* This enzyme rapidly oxidises both o- and ρ-dihydroxyphenols. Laccase does not hydroxylate monophenols, are more soluble and significantly more resistant to SO_2. The bisulphite ion species (HSO_3^-) helps to protect juices and wines from oxidative browning reactions, as well as scavenge hydrogen peroxide formed from via oxidation of phenolic molecules. In the presence of catalysers, SO_2 binds with dissolved O_2 thus acting as a reducing agent. Thus, it protects the wine from chemical oxidation and enzymatic oxidation. The oenological importance of SO_2 applies especially to white wine, in which case the wines would lose some of their important characteristics if they were not protected by SO_2 (Usseglio-Tomasset, 1992; Zoecklein *et al.*, 1995; Ribéreau-Gayon *et al.*, 2000a, b).

In wine, SO_2 is in a pH-dependent equilibrium consisting of bound SO_2, and free SO_2, the latter being made up of molecular SO_2, and bisulphate (HSO_3^-) and sulphite ($SO3^{2-}$) ions. **Fig. 2.5** depicts the different fractions of sulphur that exist in wine. Together these forms represent the total level of SO_2 (Wibowo *et al.*, 1985). In red wines, the major compounds that bind with SO_2 are carbonyl compounds, in particular acetaldehyde, sugars, pyruvate and anthocyanins. Glucuronic and galacturonic acids resulting from hydrolysis of plant cell walls also binds with SO_2. Several sugars present in must reacts with SO_2. Bound SO_2 has little inhibitory effect against most yeasts and AAB, but 50 mg/L bound SO_2 is believed to be inhibitory against LAB (Stead, 1993; Zoecklein *et al.*, 1995). A free SO_2 level of 1 to 10 mg/L is sufficient to inhibit LAB growth (Rankine & Bridson, 1971; Somers & Westcombe, 1982). Free SO_2 in wine is responsible for the anti-microbial free fraction, which is at an optimum at pH 3.5 and below.

Excessive concentrations of SO_2 should absolutely be avoided for health reasons, could also interfere with wine bouquet and can also be a source of hydrogen sulphide (H_2S) formation (Gerbaux *et al.*, 1997). Zoecklein *et al.* (1995) reported about the industry-wide trend toward reducing SO_2 based on public health concerns, better fruit quality, desire for MLF and the perceived elegance of wines.

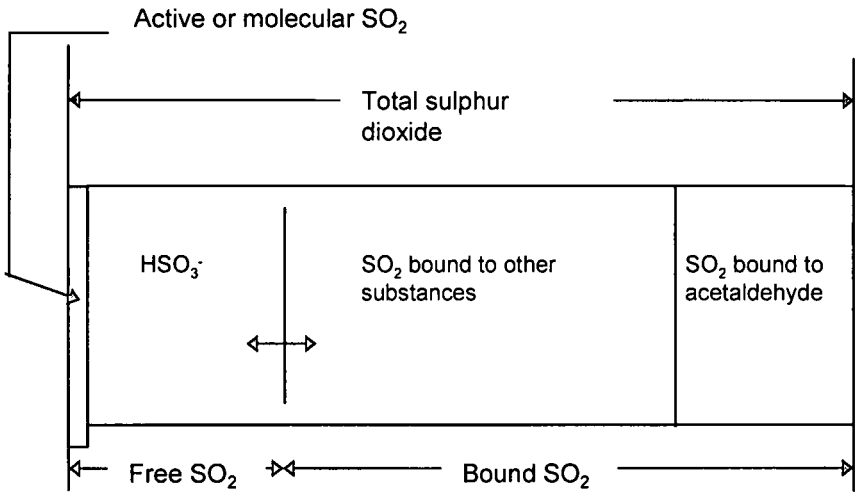


Figure 2.5 Total and bound SO_2 diagram (adapted from Ribéreau-Gayon *et al.*, 2000a).

Higher concentrations of SO_2 give wine a suffocating and irritating odour and burning sensation (Ribéreau-Gayon *et al.*, 2000a, b) as well as impart a metallic and harsh character (Zoecklein *et al.*, 1995). Furthermore, excessive levels of free SO_2 add a pungent aroma, sharpness in the nose and a "soapy" smell (Zoecklein *et al.*, 1995). Vally & Thompson (2001) studied self-reporting wine sensitive asthmatic subjects. They only found four of 23 persons sensitive to sulphites at levels of 300 parts per million and that it could not be concluded that sulphites in wine are solely responsible for allergic and asthmatic reactions (Vally & Thompson, 2001).

The precise adjustments of SO_2 levels are complicated because of the complex chemical equilibrium of this molecule in wine. SO_2 permits the storage of many types of wine known, today that would not exist without its protection. It also permits extended barrel maturation and bottle ageing (Ribéreau-Gayon *et al.*, 2000a, b).

2.4.2 DIMETHYLDICARBONATE AND DIETHYLDICARBONATE

Dimethyldicarbonate (DMDC; also known as dimethylpyrocarbonate or DMPC) is the methyl analogue of diethyldicarbonate (DEDC or diethylpyrocarbonate). DEDC's use in wine has since 1972 been banned by The Food and Drug Administration (FDA) of the United States of America (USA) due to reports of ethyl carbamate (urethane), an animal carcinogen, being formed under certain conditions which is caused by the decomposition of this sterilant in wine. Both DMDC and DEDC are active in the inhibition of yeast at relatively low levels of addition (< 250 mg/L) and are usually added prior to bottling in the

soft-drink industry, in brewing, and in winemaking. DMDC is a colourless volatile liquid with faint to no odour. It has a molecular weight of 134.45 g/mol and specific gravity of 1.26 (Ough, 1975b; Stafford & Ough, 1976; Peterson & Ough, 1979; Porter & Ough, 1982; Ough *et al.*, 1988; Zoecklein *et al.*, 1995; Delfini *et al.*, 2002). However, Delfini *et al.* (2002) suggested that DMDC should be added to grape must or wines in as pure form as possible. The commercial product of DMDC in South Africa is Velcorin® (Bayer Industrial Chemical Division).

Delfini *et al.* (2002) reported that *S. cerevisiae* was resistant up to 200 mg/L DMDC, whereas S. *bayanus* and *S. uvarum* were inhibited at 150 mg/L. The most common yeasts generally found in un-inoculated grape must, *Kloeckera*, *Candida*, and *Pichia*, can be controlled with 200-250 mg/L DMDC. The most resistant yeasts species were inhibited at 500 mg/L DMDC. 500 mg/L DMDC killed 50% of the *Acetobacter aceti* and 100% of the *Lactobacillus* species. A complete sterilisation can be accomplished by adding 1 g/L DMDC to must or wine (Delfini *et al.*, 2002) (Ough, 1975b; Stafford & Ough, 1976; Peterson & Ough, 1979; Porter & Ough, 1982; Ough *et al.*, 1988; Zoecklein *et al.*, 1995; Delfini *et al.*, 2002).

The activity of DMDC is less effective against bacteria. Porter & Ough (1982) showed that a 100 mg/L DMDC was enough to have an effective death rate on yeast counts which was zero after 10 minutes. In the study of Delfini *et al.* (2002) it was shown that that one hour of contact with 10 g/L DMDC or two hours of contact with 5 g/L DMDC was sufficient to sterilise must. Thus, it would be possible to lower concentrations of DMDC by using longer exposure times. Grape must treated with 200 mg/L DMDC should not be inoculated with a selected yeast strain for at least 12 hours (Delfini *et al.*, 2002).

The reactivity of DMDC is similar to that of an acid chloride, being subject to attack by any nucleophilic reagent containing a suitable active hydrogen atom: H_2O, alcohols, carboxylic acids, phenolics, amines, thiol groups etc. The mechanism of inhibition is the hydrolysis of yeast glyceraldehydes-3-phosphate dehydrogenase and alcohol dehydrogenase yielding inactive forms of the enzymes. Ough (1975b) reported that DMDC did not contribute to any off-flavours and could not be detected by judges at a concentration of 200 mg/L. In addition, the methanol levels found by decomposition of DMDC was not toxicologically significant. Porter & Ough (1982) showed that the optimum temperature for the maximum effectiveness of DMDC was slightly above 20°C and that the sterilant was more effective in higher alcohol concentrations, thus indicating a synergistic effect between higher alcohol concentrations and higher temperatures. Ough *et al.* (1988) showed that yeast and LAB re-growth in bottled wine can be controlled by judicious use of DMDC and SO_2, thus resulting in lowered amounts of the two additives for microbiological control in wines at bottling.

DMDC in wine reacts with alcohols to form breakdown products of methanol, CO_2 and methyl alkyl carbonates. Methyl alkyl carbonate formation is linear with both DMDC and alcohol concentrations. Peterson & Ough (1979) found that the possible by-products of DMDC reaction with higher alcohols are of little significance. Methanol is known to be toxic

to humans when taken orally at 340 mg/kg of body weight. The level of methanol formation is directly proportional to DMDC addition, but appears to be independent of pH and ethanol concentration. The ethanolyis of DMDC will produce ethyl methyl carbonate. Some authors found that the formation of ethyl methyl carbamate is directly proportional to the level of DMDC added. The reaction with ammonia yields methyl carbamate and has been found to be non-carcinogenic. The ethyl methyl carbonate residues, a stable and proportional derivative of DMDC in hydroalcoholic solutions in wine, can be determined using conventional extraction and gas chromatographic techniques, thus indicating the amount of DMDC initially added. The OIV approved a maximum dose of 200 mg/L DMDC in wine but not in must. The USA and New Zealand currently permit the addition to wine of up to 400 mg/L DMDC (Ough, 1975b; Stafford & Ough, 1976; Peterson & Ough, 1979; Tegmo-Larsson *et al.*, 1989; Zoecklein *et al.*, 1995; Delfini *et al.*, 2002).

South Africa's maximum limit in wine and must is a 100 mg/L (http://www.sawis.co.za). It is not yet clear if DMDC affects growth factors such as ascorbic acid, total amino acid, fructose, glucose, lycopene and β-carotene (Ough, 1975b; Stafford & Ough, 1976; Peterson & Ough, 1979; Tegmo-Larsson *et al.*, 1989; Zoecklein *et al.*, 1995; Delfini *et al.*, 2002).

2.4.3 FUMARIC ACID

According to Zoecklein *et al.* (1995) fumaric acid is a relatively strong organic acid and its addition may result in a decrease of pH and increase in titratable acidity. Tchelistcheff *et al.* (1971) found that fumaric acid inhibited MLF conducted by *Leuconostoc citrovorum* ML-34 in three tested cultivar wines. They confirmed that fumaric acid, at a level of 600 mg/L, would be very likely to prohibit MLF for more than a year in various wine types. However, each wine type should be tested first. Low concentrations of fumaric acid may enhance MLF through fumarase-generated malic acid (Tchelistcheff *et al.*, 1971).

Fumaric acid may be utilised by yeast and is thus added to wine after the first racking. Disadvantages of fumaric acid include: i) difficulty in dissolving; ii) having its own unique sensory properties; and iii) possible use as a carbon source by LAB if concentrations of fumaric acid is inadequate for LAB inhibition. The effectiveness of fumaric acid is enhanced by a low pH, adequate SO_2 levels (e.g. 50 mg/L free SO_2) and low numbers of spoilage microorganisms (Zoecklein *et al.*, 1995). Fumaric acid is not registered as a legal preservative in South Africa (http://www.sawis.co.za).

2.4.4 CITRIC ACID

Citric acid is used in the food industry to inhibit the growth of *C. botulinum* due to its Ca^{2+} chelating activity (Brul & Coote, 1999). Citric acid is used in wine to inhibit yeast growth, however LAB are able to use citric acid as part of their metabolism and cause a marke increase in the VA content. Studies have shown that adding citric acid to a laboratory medium stimulates the growth rate and enhances the growth yield of LAB. It seems that

citric acid is involved in the biosynthesis of the aspartate-derived essential amino acids and glucose in the cysteine biosynthesis: asparagines and isoleucine. It was also made clear that it is undoubtedly an advantage to use *O. oeni* strains that do not metabolise citrate under wine conditions (Saguir & Manca de Nadra, 2002; Rozès *et al.*, 2003). Citric acid is legal for use in wine in South Africa; however no legal maxims are indicated (http://www.sawis.co.za).

2.4.5 PIMARICIN/NATAMYCIN

Natamycin ($C_{33}H_{47}NO_{13}$) is an antifungal antibiotic that is permitted in some countries for surface preservation of cheese but is not active against bacteria (Russel *et al.*, 1991). Natamycin is produced through a selected strain of *Streptomyces natalensis* (www.primepharma.co.za). JianFen & Yong (2004) found that 2.5 µg/mL to be a minimum inhibitory concentration of natamycin when tested on yeasts. Their research indicated that natamycin was more effective when added at the lag phase rather than the stationary phase of yeast growth. Pimaricin is according to Ribéreau-Gayon *et al.* (2000b) auto degradable and has a fungistatic effect without any known secondary effects. It is used with success in the food industry. The legal maxim levels found in South Africa is 30 mg/L (http://www.sawis.co.za). The locally antimicrobial agent used in South Africa is Delvocid® which contains natamycin and glucose. Delvocid® is used in wine or must to inhibit fungal and yeast growth and is normally added to wine or must at a concentration of a 100 mg/L. Pimaricin is according to www.primepharma.co.za a 50%/50% blend of lactose/glucose and natamycin. At 7ºC it takes two to four days for 50% of the Pimaricin introduced into wine to break down. Therefore after 7-14 days all the Natamycin will have disappeared (www.primepharma.co.za).

2.4.6 SORBIC ACID

Sorbic acid is a short-chained unsaturated fatty acid widely used in the wine and food industry (Stead, 1993; Zoecklein *et al.*, 1995; Xie *et al.*, 1999; Brul & Coote, 1999; Ribéreau-Gayon *et al.*, 2000b). It is usually sold as a soluble salt in the form of potassium sorbate. The legal sorbic acid and potassium benzoate limit in South Africa is 200 mg/L (http://www.sawis.co.za). Due to sorbic acid's fungistatic nature it is used as an effective inhibitor against fermentative yeast. As preservative in food it inhibits the germination and outgrowth of bacterial spores and outgrowth of fungal cells. It has little inhibitory effect towards LAB, AAB and oxidative film forming yeast (Stead, 1993; Zoecklein *et al.*, 1995; Brul & Coote, 1999; Ribéreau-Gayon *et al.*, 2000b).

Sorbic acid must be incorporated into the cell to be effective. The antimicrobial activity resides in the undissociated molecule. Once incorporated into the cell the sorbic acid may be operative against the dehydrogenase enzyme system of yeasts and moulds, interfering with oxidative assimilation of carbon. Sorbic acid's effectiveness is dependent on several parameters which includes pH, SO_2, alcohol content and yeast genera and species

present. An elevated pH results in the reduced effectiveness of sorbic acid, where SO_2 appears to operate in a synergistic manner with sorbic acid. Sorbic acid has an inhibitory activity at low pH because this favours the uncharged, undissociated state of the molecule which is freely permeable across the plasma membrane and thus able to enter the cell. Inhibition of growth by sorbic acid has been proposed to be due to a number of actions including, membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis and the accumulation of toxic anions (Zoecklein *et al.*, 1995; Brul & Coote, 1999; Ribéreau-Gayon *et al.*, 2000b).

Sweet wines with higher alcohol content require less sorbic acid for stabilisation than wines with lower alcohol content. Sorbic acid is usually added after bottling. Benzoic acid, as potassium or sodium benzoate, is a permissible additive in addition to sorbate when added to coolers. The OIV limit for sorbic acid is 200 mg/L. Wines treated with sorbic acid should be stored at low O_2 conditions with high enough levels of SO_2 to prevent the growth of LAB and AAB. The use of potassium sorbate in wines destined for long-term ageing is not recommended due to a lack of effectiveness and the appearance of unwanted tastes (Zoecklein *et al.*, 1995; Brul & Coote, 1999; Ribéreau-Gayon *et al.*, 2000b).

Certain LAB are able to reduce sorbic acid to sorbinol through hydrogenation. Thereafter, under wine conditions, it will isomerise to form the alcohol 3,5-hexadiene-2-ol. This alcohol reacts with ethanol to form 2-ethoxyhexa-3,5-diene, which is responsible for the "geranium tone". This phenomenon has been observed only in oenococci. Care should thus be taken when adding sweeteners or treating wine with sorbic acid as preservative, since auto-oxidation can take place resulting in products such as acrolein, crotonaldehyde and formic acid. Another off-odour, that of 'pineapple-celery', has occurred in sparkling wines from the esterification of sorbic acid to produce ethyl sorbate (Stead, 1993; Du Toit & Pretorius, 2000).

2.4.7 BENZOIC ACID

According to Zoecklein *et al.* (1995) the use of benzoic acid as the potassium or sodium salt has been restricted to the food industry in the USA. It is, however, approved for the use in wine coolers with a legal limit of 1 mg/L. The maxiumum level of sodium benzoate in South Africa is 200 mg/L (http://www.sawis.co.za). Benzoic acid is used in combination with sorbic acid and SO_2. The antimicrobial activity of benzoic acid is linked to the unionised form and is most effective at pH 4.2 and lower. As the pH decreases, the amount of benzoic acid needed for inhibition also decreases (Zoecklein *et al.*, 1995).

Some authors reported that quinic acid antagonised the inhibitory effect of potassium sorbate and sodium benzoate, thus resulting in increased additions of preservatives to ensure microbiological stability (Stead, 1994).

2.5 PHYSICAL STABILISATION AND STERILISATION

2.5.1 HEAT/PASTEURISATION

Zoecklein *et al.* (1995) pointed out the fact that hot (> 82°C) H_2O or steam are ideal sterilants due to their penetrative properties that are active against all wine/juice microorganisms. The advantages of steam are that its non-corrosive nature leaves no residues and it is relatively inexpensive. Steam or hot H_2O can be used at the bottling line and to sterilise tanks (Zoecklein *et al.*, 1995).

Ribéreau-Gayon *et al.* (2000b) explained the use of heating of wine to denaturate the unstable proteins in white wines, to reduce copper in the form of colloidal copper sulphide and for biological stabilisation. Pasteurisation was initially used to protect wine from microbial spoilage caused by AAB and LAB. Microbial spoilage can be avoided by other means such as careful fermentation management, SO_2 and the reduction of contaminant populations by various clarifications processes. Although wine may be pasteurised in the bottle or just before bottling, there are other stabilisation techniques such as sulphuring and sterile filtration that is easier to use. The temperature and heating time required depend on the aim of the treatment. Normal pasteurisation consists of passing wine through a dimpled plate heat exchanger. The wine circulates in very thin layers and is heated (60-65°C) by hot H_2O which moves in the opposite direction, on the other side of the plates. The heated wine is cooled on leaving the exchanger by circulating it through circuits cooled by wine that is about to be treated (Ribéreau-Gayon *et al.*, 2000b).

Flash pasteurisation entails heating of wine to 90°C for a few seconds and then cooling it rapidly in a high-performance plate heat exchanger. It is considered that this high speed process is less likely to affect the wine's organoleptic characteristics. The heating of bottled wine to 60°C ensures that all germs are killed and subsequently prevents further contamination. However, it is not as widely used for wine as for beer due to wine's inherent natural stability. The main heat treatment for wine is high temperature bottling. The principle consists of heating wine to the relatively moderate temperature required to destroy yeast (45-50°C, depending on the alcohol content and the possible presence of sugar). The hot wine is transferred directly into bottles, sterilising both glass and cork as it cools. The equipment is easily installed on a standard bottling line. In view of the risk of even slight organoleptic changes, this technique is more suitable for average quality wines than fine wines. This treatment should only be used for wines that have been stabilised in terms of colloidal turbidity, especially protein and copper casse, as these problems would otherwise be likely to be triggered by heating. A replacement to high temperature bottling is fine filtration processes to achieve absolute sterility at cool temperatures, provided that perfect hygiene is maintained throughout the bottling system (Ribéreau-Gayon *et al.*, 2000b).

2.5.2 CENTRIFUGATION

Centrifugation is defined by accelerating settling of sediment in juice or wine by rotating it very fast around an axis. The sediment moves away from the axis due to centrifugal force. At the same time, the gravitational force is multiplied by a considerable factor, proportional to the speed of rotation squared. The volume of liquid treated is restricted to the capacity of the system, but this limitation can be overcome by using continuous centrifuges. High performance centrifuges have the advantage over standard centrifuges due to its higher rotation speeds and are even capable of eliminating the lightest particles such as bacteria. Centrifugation is a rapid method for obtaining wines that are clean, stable and ready to drink and minimises losses of lees wine (Ribéreau-Gayon *et al.*, 2000b). In the study of Ferrando *et al.* (1998) they showed that centrifugation combines the advantages of settling and vacuum filtration, since it is a continuous process that generates a fairly low amount of solid residue, hence reducing environmental problems and maintaining wine quality.

Schauz (1996) explained that centrifugation can be used with great effect to remove 99% of yeast cells to obtain sweeter wines as well as the removal of harmful microorganisms and fungicide residues. However, these practices must operate under sufficient anaerobically conditions to avoid excessive oxidation. Practical uses of centrifugation include clarification of must after pressing, during and at the end of fermentation, wines after fining and facilitating tartrate precipitation (Ribéreau-Gayon *et al.*, 2000b).

2.5.3 FILTRATION

Filtration is a separation technique used to eliminate a solid in suspension from a liquid by passing it through a filter medium consisting of a porous layer that traps the solid particles. 'Filtering' generally refers to the clarification of a liquid, while 'filtration' is more used to describe the technical process. There are several types of filtration defined by using different filter media which includes: i) diatomaceous earth precoat (kieselguhr); ii) cellulose sheets or lenticular modules; iii) synthetic polymer membranes; and iv) inorganic or organic membranes (Ribéreau-Gayon *et al.*, 2000b).

The more common type of membrane used for microfiltration and ultrafiltration is composed of organic polymers (cellulose acetate, polyethylene, polyamides or polysulphone). However, inorganic membranes of the ceramic type are being used more due to their high resistance to chemical degradation (Palacios *et al.*, 2002). Filtration through fine filter media leads to rapid clogging, whereas, if the medium is too coarse, all the particles are not removed (Ribéreau-Gayon *et al.*, 2000b).

The main factors responsible for clogging when using microfiltration include adsorption of filtrate, deposition of solids and chemical interaction between membrane material and colloid components of wine (Carneiro *et al.*, 2002; Palacios *et al.*, 2002; Gergely *et al.*, 2003). Carneiro *et al.* (2002) proposed enzymatic hydrolysis prior to membrane filtration to minimise clogging when working with fruit juices. Gergely *et al.* (2003) described a multi-

objective optimisation method to achieve high quality wines using membrane filtration. They explained that a high filtration flux is desirable for economic reasons.

Wines that are bottled relatively young are subjected to a greater number of clarification operations. Sheet, lenticular module (flat-sheet filters mounted on tray filters or built into closed filters that prevent leaks) or membrane filtration (synthetic membranes with calibrated pores) can be used to obtain wines with low microbe levels or even totally sterile wines (Ribéreau-Gayon *et al.*, 2000b). However, Ubeda & Briones (1999) found spoilage microorganisms in some wines after filtering and thus the need for rigorous microbiological control in cellars is essential to ensure the biological stability of wine once it has been bottled.

In membrane filtration, particle retention limits the duration of the filtration cycles by cumulative clogging and when the feed stream contains a high concentration of solids (>0.5%), the use of microfiltration is recommended in the crossflow configuration (Palacios *et al.*, 2002). Ribéreau-Gayon *et al.* (2000b) explained that membrane characteristics include separation efficiency; high permeate flux and good physical, chemical and heat resistance. Microfiltration membranes consist of a thin filter layer deposited on a base of the same (asymmetrical membranes) or a different type (asymmetrical and composite membranes) of materials (Ribéreau-Gayon *et al.*, 2000b).

Crossflow microfiltration can be used satisfactory in all clarification and stabilisation steps of industrial winemaking, which includes clarification of must settlings, clarification of wine lees or partially fermented lees, microbiological stabilisation of must and physical-chemical stabilisation of wine (Palacios *et al.*, 2002).

The effectiveness of filtration processes may be assessed by measuring various parameters indicative of clarity. These parameters include turbidity, solid content, particle counts and microbiological analyses of the wine. Two parameters define the performance of a filter medium: porosity and permeability. Porosity expresses the percentage of empty space in a porous structure in relation to total volume. Increasing porosity of the filter increases the capacity to retain contaminants. Permeability describes the property of a filter medium to let liquid through at higher or lower speeds. Filtration is known to have potentially harmful effects and is particularly criticized for making wines thinner. However, properly controlled filtration has positive effects on quality, whereas careless or excessive treatment may have a negative impact (Ribéreau-Gayon *et al.*, 2000b).

Palacios *et al.* (2002) explained that crossflow microfiltration of wines gave a superior quality of clarification to other filtration techniques (conventional filtration), mainly from the point of view of microorganisms retention. Moreover, microfiltration did not modify in a significant way the sensory qualities of wines in comparison to the common techniques of clarification (Palacois *et al.*, 2002). Carneiro *et al.* (2002) also found that microfiltration has the advantage, in relation to the thermal processes, of using mild temperatures and pressure conditions to retain the nutritional quality and the sensorial attributes of fruit juices. Gergely *et al.* (2003) found that membrane filtration decreased the depth of red colour of red wine, but the significant increase in the quality resulted in higher average

sensorial evaluation of the wines. They concluded that the properly chosen membrane filtration did not have any negative effect on the organoleptic characteristics of the wines, while it clarified, stabilised and sterilised the wines.

Ultrafiltration has the disadvantage of low filtration yields and by removing some colloidal and phenolic fractions that have a positive influence on the sensorial characteristics of the product (Palacios *et al.*, 2002).

Overall, contact with O_2 during filtration should be prevented which could cause ferric taint or a loss in aroma and free SO_2. Poor quality filter media may also transmit an earth, paper or cloth taint to the wine, although only the first few litres of wine are affected. However, some researchers found that filtration did not have any negative effect on chemical composition of wine, regardless of by using diatomaceous earth precoat, clarifying or sterilising flat sheet filters or membrane filters (Ribéreau-Gayon *et al.*, 2000b).

2.6 BIOPRESERVATION

2.6.1 BACTERIOCINS

2.6.1.1 General

Bacteriocins are defined as biologically active protein moieties with bactericidal action and are ribosomally synthesised. Bacteriocins are clearly distinguishable from clinical antibiotics in terms of application, synthesis, activity, host cell immunity, mechanism of target cell, resistance or tolerance, interaction requirements, mode of action and toxicity/side effects. Bacteriocins differ in terms of size, microbial targets, modes of action and immunity mechanisms. Bacteriocins have also been grouped into different classes based on molecular weight, structure, stability and interaction with membranes of antagonised cells. Due to its narrow killing spectrum it's only toxic to bacteria closely related to the producing strain, thus differing from traditional antibiotics. Bacteriocins are heat resistant and stable for several months, but are inactivated by proteolytic enzymes such as trypsin, pepsin and other proteases, such as gastric proteinases. Nomenclature of bacteriocins is generally based on the genus or species of origin, e.g. lactococcin from *Lactococcus lactis* or monocin from *Listeria monocytogenes*. The most intensively studied bacteriocins, the colicins, are produced by *Escherichia coli*. The colicins constitute a diverse group of antibacterial proteins, which kill closely related bacteria by various mechanisms such as inhibiting cell wall synthesis, permeabilising the target cell membrane, or by inhibiting RNA or DNA activity. Bacteriocin molecules range from large domain-structured proteins, such as colicin, to small peptide lantibiotics, such as nisin. The major classes of bacteriocins produced by LAB include: (I) lantibiotics (19 to 50 amino acids), (II) small heat stable peptides (non-lanthionine containing membrane-active peptides), (III) large heat stable proteins, and (IV) complex proteins whose activity requires

the association of carbohydrate or lipid moieties. Nisin A, the best studied lantibiotic, is produced by *L. lactis* and strongly inhibits the growth of a wide range of Gram-positive bacteria. Nisin consist of cationic and hydrophobic peptides that form pores in target membranes. Pediocin PA-1 from *Pediococcus acidilactici* is part of the class II bacteriocins. The class II bacteriocins are further divided into three subgroups (Abee *et al.*, 1995; Dykes 1995; Daw & Falkiner, 1996; Schillinger & Holzapfel, 1996; Helander *et al.*, 1997; Gänzle *et al.*, 1999; Riley & Gordon, 1999; Konings *et al.*, 2000; Cleveland *et al.*, 2001; Guerra *et al.*, 2001; Guerra & Pastrana, 2002; Héchard & Sahl, 2002; Le Roy *et al.*, 2002; O'Sullivan *et al.*, 2002; Riley & Wertz, 2002).

The action and production of bacteriocins against sensitive microorganisms are influenced by factors such as pH, temperature, cell concentration, lipid content, proteolytic enzymes, liquid vs. solid system, redox potential and composition of the nutrient media. In food matrices the bacteriocin activity may be affected by (i) changes in solubility and charge of bacteriocins, (ii) binding of bacteriocins to food components, (iii) inactivation by proteases, and (iv) changes in the cell envelope of the target organism as a response to environmental factors. The effiency of nisin Z against *L. monocytogenes* are significantly reduced in the presence of di- and trivalent cations such as Mg^{2+}, Ca^{2+} or Cd^{3+}, thus indicating that these di- and trivalent ions in foods could potentially reduce the efficiency of nisin against Gram-positive spoilage bacteria and pathogens (Abee *et al.*, 1995; Helander *et al.*, 1997; Gänzle *et al.*, 1999; Guerra & Pastrana, 2002).

Nisin are commercially available as a biopreservative (Brul & Coote, 1999) and has GRAS status (O'Sullivan *et al.*, 2002).

2.6.1.2 Mode of action

Bacteriocins are either bacteriostatic (inhibits bacteria) or bactericidal (kills bacteria). Bacteriocin activity is directed against the cell membrane through adsorption to specific receptors located at the external surface of sensitive cells, thus meaning that it must penetrate the cell wall of the target bacteria before the antagonistic effect can occur. Because of fundamental differences in structure between the cell walls and membranes of the different Gram-reaction of bacteria, different mechanisms are involved in the initial interaction. Gram-negative bacteria have a thinner peptidoglycan layer in their cell walls and may thus be negatively affected by larger proteins. Gram-negative bacteria possess an additional layer, the so-called outer membrane, which is composed of phospholipids, proteins and lipopolysaccharides (LPS), and this membrane is impermeable to most molecules. The presence of porins in this layer will allow the free diffusion of molecules with a molecular mass below 600 Da. The smallest bacteriocins produced by LAB are approximately 3 kDa and are thus too large to reach their target, the cytoplasmic membrane. However, studies showed that nisin caused a change in the permeability barrier of *Salmonella* species and other Gram-negative bacteria (Abee *et al.*, 1995; Dykes 1995; Daw & Falkiner, 1996; Helander *et al.*, 1997; Gänzle *et al.*, 1999).

The mode of action of bacteriocins may also vary from one type to another (Daw & Falkiner, 1996). Depletion of the transmembrane proton motive force (PMF) is a common mechanism in LAB and many other bacteria. Depletion of PMF by some bacteriocins is thought to be mediated by the formation of pores or ion channels in the bacterial membrane. These channels may result in free efflux of ions necessary for energy transduction. Since the PMF is integrally linked with energy generation by the cell, the net result is cellular death or inhibition of growth. In Gram-positive bacteria nisin has been shown to act on energised membrane vesicles to disrupt the PMF, inhibit uptake of amino acids, and cause the release of accumulated amino acids. Nisin's primary target is the cytoplasmic membrane of the bacterial cell. It seems that the greatest interaction of nisin is with the negatively charged phospholipids, followed by insertion into the membrane and pore formation leading to depletion of the PMF and efflux of small solutes. Nisin-producing strains generally encode for their own resistance (immunity) to the bacteriocin they produce. Nisin resistance may also be encoded by mobile genetic elements, usually plasmids that are not linked to bacteriocin production. Spontaneous resistance to bacteriocins may occur at low levels in natural bacterial populations. Such mutants are at a selective advantage in populations exposed to high levels of bacteriocins. It is recommended that a combination of bacteriocins is used to reduce resistance. Nisin has also been shown to act on *Clostridium* and *Bacillus* spores. Some Gram-positive bacteria have been shown to be resistant to nisin due to their ability to synthesise an enzyme, *nisinase*, which could inactivate nisin. This enzyme was also isolated from several *Bacillus* spp. Other modes of action include inhibition of DNA synthesis and induced DNA degradation (Abee *et al.*, 1995; Dykes 1995; Daw & Falkiner, 1996; Gänzle *et al.*, 1999; Konings *et al.*, 2000; Héchard & Sahl, 2002). Several modes of action are also discussed in Cleveland *et al.* (2001).

Guerra & Pastrana (2002) explained that the differences in sensitivity and resistance of strain to the different bacteriocins are related to the existence of different, but specific, surface receptors for different bactercions in Gram-positive bacterial cells.

2.6.1.3 Applications of bacteriocins winemaking

Researchers found that it is better to employ bacteriocins with a high specific activity range (Abee *et al.*, 1995). Nisin A and Z displays increased activity at acidic pH and is active at wine pH. MLF is favoured in most red wine and unfavourable in fruitier white wines. The addition of nisin after MLF will protect wine against later spoilage by any remaining LAB (Radler, 1990a, b).

Nisin could also have an application in the fermentation industry dealing with the production of fruit brandies (Delves-Broughton, 1990). LAB isolated from wine were successfully inhibited by nisin without affecting yeasts performing the AF at an experimental scale level. When nisin was used as a preservative against LAB in small-scale fermentations, no influence was detected on the sensory quality of wine (Radler, 1990a, b). Chung & Hancock (2000) showed that the combination of lysozyme and nisin

was more effective than the individual agents alone and could thus be an option for addition in wine. Presently, the use of nisin in wine is prohibited.

2.6.2 GLUCOSE OXIDASE

Glucose oxidase catalyses the oxidation of β-D-glucose to δ-D-gluconolactone (Gibson *et al.*, 1964), but glucose oxidase are also able to attack 2-deoxy-D-glucose, D-mannose, D-galactose and D-xylose (Keilin & Hartree, 1948). The enzymatic reaction can be divided into two steps: (i) the oxidation of β-D-glucose yielding hydrogen peroxide (H_2O_2) and δ-D-gluconolactone and (ii) the non-enzymatic hydrolysis of δ-D-gluconolactone to gluconic acid. If the catalase is present, the H_2O_2 can be reduced to H_2O and molecular O_2 (Ough 1975a). Glucose oxidase is formed by *Aspergillus niger* and *Penicillium* spp. and has GRAS status (Fugelsang *et al.*, 1995; Malherbe *et al.*, 2003).

Glucose oxidase can be used for the deoxygenation of wine and stabilisation against both browning and organoleptic changes. Glucose oxidase wines have a higher SO_2 binding power, but are more stable against browning. This could be due to an increase in quinine production and the regeneration of oxidisable phenolic substances. However, large-scale use has never occurred. One of the reasons for limited use in wine is the inability of fungal catalase to function catalytically in the presence of ethanol. Without catalase activity, glucose oxidase still deoxidises the product, but it is in reality an effective H_2O_2 producer until glucose or O_2 is depleted or the enzyme is inactivated. However, the presence of SO_2 and/or ascorbic acid is needed to scavenge the peroxide formed by glucose oxidase, resulting in low O_2 permeation into the finished product, resulting in a indirectly affect on strictly aerobic microorganisms owing to O_2 depletion (McLeod & Ough, 1970; Temple & Ough, 1975; Fugelsang *et al.*, 1995; Pickering *et al.*, 1998, 1999; Power, 1998).

2.6.2.1 Applications of glucose oxidase in winemaking

The glucose oxidase-catalase desugaring process is used in wine for the conversion of glucose to gluconic acid to produce low/reduced alcohol wines (Power, 1998). The gluconic acid results in a pH decrease and the other secondary product, H_2O_2, inhibits the growth of spoilage organisms. The decrease in pH as well as the H_2O_2 acts as biological control agents, but the H_2O_2 is the major factor in the inhibitory effect (Yoo & Rand, 1995). However, these findings still needs to be evaluated and proven in wine.The over-expression of the glucose oxidase gene in certain microorganisms is an alternative for the increased production of glucose oxidase. The laws against the use of genetically modified organisms (GMOs) are limiting factors at the moment (Malherbe, 2002).

2.6.3 LYSOZYME

Enzymes that degrade bacterial walls from the outside, such as lysozyme, have been applied in the preservation of foods (Brul & Coote, 1999). Lysozyme, as an additive to

wine, has been approved by the OIV and the European Commission for the use in winemaking (Bartowsky, 2003). Lysozyme is a naturally occurring enzyme, extracted from hen egg white, consisting of 129 amino acid residues with a molecular weight of about 14,400 Daltons (Proctor & Cunningham, 1988; Fordras S.A.) and displays murimidase and chitinolitic activity (Board, 1995). Lysozyme is supplied as a white, odourless and non-toxic microcrystalline powder that resuspends easily in luke warm H_2O (Bartwosky, 2003). It's effective in attacking the cell wall of different bacterial Gram-positive species (Fordras S.A.) and is one of the most popular and safe bactericidal proteins (Arima *et al.*, 1997).

Lysozyme was first isolated from human nasal secretions by Alexander Fleming in 1921. Lysozyme has since been isolated in human tears, saliva and mother's milk, as well as viruses, bacteria, phage, plants, insects, birds, reptiles and other mammalian fluids. However, the most important application of lysozyme is in the cheese industry, where it is used to prevent a problem known as "butyric late blowing". This problem occurs during the ripening of certain European-type cheeses and is due to a naturally occurring, spore forming bacterial contaminant of milk, *Clostridium tyrobutyricum* (Wasserfall & Teuber, 1979, http://www.wynboer.co.za). Lysozyme is, however, not effective against AAB species or yeast, such as *Saccharomyces* or *Brettanomyces*.

2.6.3.1 Characteristics

Amphilic helix stretches in the C-terminus of T4 lysozyme mediate its bactericidal and fungistatic activities. The enzymatic activity is completely abolished by heat denaturation of lysozyme, but the antimicrobial functions remain preserved, resulting in the non-enzymatic microbicidal activity of lysozyme (Düring *et al.*, 1999). Salton (1957) explained that amino acid composition of crystalline lysozyme had an absence of sulfhydryl groups and a high content of arginine, with lysine as the N-terminal amino acid and leucine as the C-terminal amino acid.

Lysozyme has a broad spectrum between pH 6-7 and the optimum pH for lysing *Micrococcus lysodeikticus* is 6.5 (Blake *et al.*, 1965). The isoelectric point is at pH 10.5-11 (Salton 1957). Dimerisation can occur in a pH range of 5 to 9, in which the net charge changes a little while the molecule undergoes no major structural modification (Sophianopoulos & Van Holde, 1964). Lysozyme has a compact tridimensional structure including four disulfide bonds. It is a highly ordered, rigid, hydrophilic and positively charged protein (Marchal *et al.*, 2002). In solution, lysozyme is relatively stable at pH 3-4 and is active over the temperature range of 1°C to near boiling point (Gao *et al.*, 2002).

2.6.3.2 Mode of action

Lysozyme (EC 3.2.1.17) is officially described as *N*-acetylhexosaminodase and is classified as a muridase (Wilkinson & Dorrington, 1975). Lysozyme contributes to bacterial killing by degradation of peptidoglycan (Ohno & Morrison, 1989). Lysozyme cleaves the β (1-4)-glycosidic bond between *N*-acetylmuramic acid (MurNAc) and *N*-acetyl-D-glucosamine (GlcNAc) in the polysaccharide, which form peptidoglycan, the essential

constituent of bacterial cell walls. This glycosidic cleavage is considered to be the first step in the lysis of bacterial cell walls. The lysing of the peptidoglycan by lysozyme leads to the disruption of the cell walls and the death of the cells due to osmotic shock. Lysozyme also cleaves the β (1-4)-linked oligosaccharides of *N*-acetylglucosamine $(GlcNAc)_n$ (Davies *et al.*, 1969; McKenzie & White, 1991; Fugelsang *et al.*, 1995; Appendini & Hotchkiss, 1997; Brul & Coote, 1999; Pilatte *et al.*, 2000).

Lysozyme has an antimicrobial action on Gram-positive bacteria. The differences in the bactericidal action between Gram-positive bacteria and Gram-negative bacteria are due to the cell envelope of the latter. This outer membrane consists of lipopolysaccharide, phospholipids, lipoprotein and protein. Gram-negative bacteria may be susceptible to cell-wall-degrading enzymes once the outer membrane has been destroyed by physical or chemical treatments. Gram-negative bacteria can be sensitised to the action of lysozyme by adding EDTA. However, EDTA is not permissible in winemaking. Several authors have reported on the synergistic action of lysozyme, nisin and citric acid to inhibit Gram-positive bacteria *L. monocytogenes* and *Listeria innocua* in foods (Andrews & Asenjo, 1987; Hughey *et al.*, 1989; Fugelsang *et al.*, 1995; Appendini & Hotchkiss, 1997; Arima *et al.*, 1997; Brul & Coote, 1999; Fordras S.A.).

2.6.3.3 Lysozyme sources

Lysozyme is found in many animal secretions with the highest concentrations occurring in tears. Hen egg white is the major source for commercial extraction. The classic method of extraction involves the absorption on bentonite and the elution of inactive contaminating proteins with phosphate buffer and aqueous pyridine. The elute was then dialysed and freeze dried. The improved method includes crystallising lysozyme as the salt of several acids and directly from egg white using 5 % NaCl. Many other methods developed to isolate lysozyme have used adsorption on chromatography columns. Others developed a cation-exchange chromatography to separate lysozyme from egg white that still retained high functionality (Proctor & Cunningham, 1988).

2.6.3.4 Inhibition spectrum

Hughey & Johnson (1987) reported the antibacterial effect of lysozyme against selected strains of *C. botulinum* and *L. monocytogenes* when used in combination with EDTA. Salton & Pavilik (1960) studied a number of Gram-positive bacteria for varying degrees of susceptibly. Their studies included strains of *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Micrococcus*, *Sarcina*, *Sporosarcina*, *Staphylococcus* and the Gram-negative *Streptococcus*. The isolated walls from all the organisms were sensitive to lysozyme. Gao *et al.* (2002) reported that lysozyme significantly affected *L. brevis*, *P. damnosus*, *L. kunkeei* and *P. parvulus* numbers at concentration of 250 mg/L.

2.6.3.5 Applications of lysozyme in winemaking

Fugelsang *et al.* (1995) explained that the use of lysozyme in combination with other antimicrobial enzymes, such as glucose oxidase or traditional preservatives such as sorbate, ethanol, temperature and low pH may increase the microbial safety and efficiency of the enzyme. Lysozyme has basic applications in winemaking and includes (i) inhibition of MLF, (ii) control the extent of MLF, (iii) microbial stabilisation of wine after MLF, and (iv) better control of sluggish and stuck AF (Gerbaux *et al.*, 2000, Fordras S.A.).

Gerbaux *et al.* (1997) showed that lysozyme had no diverse effect on the AF rate and that the composition of the wine was similar to that of the untreated wines. Gao *et al.* (2002) confirmed these results and found that lysozyme had no effect on the growth of yeast up to a lysozyme dosage of 250 mg/L. Furthermore, they showed high efficiency in the inhibition of *L. brevis*, *P. damnosus*, *L. kunkeei* and *P. parvulus* tested strains.

Wine or must of high pH values (> 3.5) have the problem that the fraction of free molecular SO_2 diminishes resulting in potential spoilage or oxidation. Because of this, some of the reasons for sluggish AF are the proliferation of LAB before the completion of AF, often resulting in an increase of VA. Naturally the risk of VA development is higher in high pH wines (Gerbaux *et al.*, 2000; http://www.lysozyme.com). Higher VA levels are due to LAB that consume glucose and fructose after malic acid, resulting in depreciation of the quality of wine (Gerland & Vialatte; Du Toit & Pretorius, 2000). Gerbaux *et al.* (1997) emphasised that the antioxidative use of SO_2 should and cannot be ignored. It would be advisable to use lysozyme in combination with SO_2.

Gerbaux *et al.* (1997) further indicated that 500 mg/L lysozyme had no effect on the colour intensity of red wine. Preliminary results also indicate that there were no differences between lysozyme treated and untreated wines except for cultivars struggling to achieve good colour during vinification such as Pinot noir (Lagarde, personal communication).

Marchal *et al.* (2002) found that the addition of lysozyme in must raised the foamability of Pinot noir champagne by 21%, when added prior to bentonite treatment. However this effect seems to differ between cultivars. Lysozyme also seems to have a protective effect on the endogenous wine proteins originating from the grape berry and yeast. At pH~3 lysozyme bears a positive net charge and is more easily adsorbed by bentonite addition. These results confirmed those obtained by Gerbaux *et al.* (1997) who found that the antibacterial effect was decreased by bentonite. Marchal *et al.* (2000) reported that crude tannins may cause significant loss of activity of lysozyme.

The action of lysozyme is immediate, and after few hours, the lysozyme is inactive (white wines) or eliminated by flocculation with tannins (red wines) (Gerland & Vialatte). However, much more research is needed to explain how lysozyme interacts with different types of phenols (e.g. catechins, epicatechins, procyanidins, polyphenols, ellagi tannins, condensed tannins etc.).

2.7 CONCLUSIONS

It is clear that LAB play an essential part in the food and beverage industry. MLF and aroma modification is the best known positive effects of LAB in wine, however the negative aspects preceeds the former. There are considerable knowledge about the possible negative effect of *Lactobacillus*, *Leuconostoc* and *Pediococcus* species on wine production and quality. Overall, for desired growth and MLF by *O. oeni* it is optimum to have a low pH (< 3.5), a temperature of 20-25°C, small amounts of SO_2 (< 10 mg/L free), no or little pesticide residues and an alcohol level below 13.5 %.

Chemical and biological preservatives possess positive and negative aspects in wine. Sterile filtration can remove microorganisms at a pore diameter of 0.45 µm, but is only applicable to yeast removal. The negative aspect of sterile filtration is the blockage of the membrane, resulting in decreased permeability and increased duration and expenses. Another area for further investigation could be the potential advantage of using bacteriocins such as nisin in combination lysozyme.

Investigations on combined treatments on gram negative bacteria such as heat or other cell wall degrading enzymes could be investigated. Further research is required to investigate the interaction between different yeast genera and species with different LAB genera and species. Due to South Africa's problem with high pH values in wines there is a need for the addition of a biological stabilisation agent due to SO_2 inefficiency at higher pH (> 3.5) values. Lysozyme can fill the need as an additional biopreservative in winemaking. A new era in terms of wine and winestyles has arrived. Presently, consumers are willing to pay more for organically made wine.

2.8 LITERATURE CITED

Abee, T., Krockel, L. & Hill, C., 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Micobiol. 28, 169-185.

Andrews, B.A. & Asenjo, J.A., 1987. Enzymatic lysis and disruption of microbial cells. TIBTECH 5, 273-277.

Appendini, P. & Hotchkiss, J.H., 1997. Immobilization of lysozyme on food contact polymers as potential antimicrobial films. Pack. Technol. Sci. 10, 271-279.

Arena, M.E., Saguir, F.M. & Manca de Nadra, M.C., 1999. Arginine, citrulline and ornithine metabolism by lactic acid bacteria from wine. Int. J. Food Microbiol. 52, 155-161.

Arena, M.E., Mancha de Nadra, M.C. & Muñoz, R., 2002. The arginine deiminase pathway in the lactic acid bacterium *Lactobacillus hilgardii* X_1B: structural and functional study of the *arcABC* genes. Gene 301, 61-66.

Arima, H., Ibrahim, H.R., Kinoshita, T. & Kato, A., 1997. Bactericidal action of lysozymes attached with various sizes of hydrophobic peptides to the C-terminal using genetic modification. *Fed.* Euro. Biochem. Soc. Lett. 415, 114-118.

Bartowsky, A. 2003. Lysozyme and winemaking. Aust. & New Zealand Grapegrower & Winemaker: Annual Technical Issue, 101-104.

Bartowsky, E.J., Xia, D., Gibson, R.L., Fleet, G.H. & Henschke, P.A., 2003. Spoilage of bottled red wine by acetic acid bacteria. Lett. Appl. Microbiol. 36, 307-314.

Beelman, R.B., Govin, A. & Keen, R.M., 1977. A new strains of *Leuconostoc oenos* for induced malol-lactic fermentation in eastern wines. Am. J. Enol. Vitic. 28, 159-165.

Blake, C.C., Koenig, D.F., Mair, G.A., North, A.C., Phillips, D.C. & Sarma, V.R., 1965. Structure of hen egg-white lysozyme. A three-dimensional Fourier synthesis at 2 Angstrom Resolution. Nature 206, 757-761.

Board, R.G., 1995. Natural antimicrobials from animals. In: Gould, G.W. (ed.), New Methods of Food Preservation. Blackie Academic & Professional, UK, pp. 40-57.

Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E., 1996. Principles and Practices of Winemaking. Chapman & Hall, New York, NY. pp. 244-273.

Britz, T.J. & Tracey, R.P., 1990. The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos*. J. Appl. Bact. 68, 23-31.

Brul, S. & Coote, P., 1999. Preservative agents in foods: mode of action and microbial resistance mechanisms. Int. J. Food Microbiol. 50, 1-17.

Buckenhüskes, H.J. 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol. *Rev.* 12, 253-272.

Campos, F.M., Couto, J.A. & Hogg, T.A., 2003. Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. J. Appl. Microbiol. 94, 167-174.

Caplice, E. & Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. Int. J. Food Micobiol. 50, 131-149.

Capucho, I. & San Romão, M.V., 1994. Effect of ethanol and fatty acids on malolactic activity of *Leuconostoc oenos*. Appl. Microbiol. Biotechnol. 42, 391-395.

Carneiro, L., Dos Santos SA, I., Dos Santos Gomes, F., Martins Matta, V. & Cabral, L.M.C., 2002. Cold sterilization and clarification of pineapple juice by tangential microfiltration. Desalination 148, 93-98.

Carreté, R., Vidal, M.T., Bordons, A. & Constantí, M., 2002. Inhibitory effect of sulphur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. FEMS Microbiol. Lett. 211, 155-159.

Chung, W. & Hancock, R.E.W., 2000. Action of lysozyme and nisin mixtures against lactic acid bacteria. Int. J. Food Microbiol. 60, 25-32.

Cleveland, J., Montville, T.J., Nes, I.F. & Chikindas, M.L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71, 1-20.

Davies, R.C., Neuberger, A. & Wilson, B.M., 1969. The dependence of lysozyme activity on pH and ionic strength. Biochim. Biophys. Acta 178, 294-503.

Davis, C.R., Wibowo, D.J., Lee, T.H. & Fleet, G.H., 1986. Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. Appl. Environ. Microbiol. 51, 539-545.

Davis, C.R., Wibowo, D., Fleet, G.H. & Lee, T.H., 1988. Properties of wine lactic acid bacteria: their potential enological significance. Am. J. Enol. Vitic. 39, 137-142.

Daw, M.A. & Falkiner, F.R., 1996. Bacteriocins: nature, function and structure. Micron. 27, 467-479.

De Beer, D., Joubert, E., Gelderblom, W.C.A. & Manley, M., 2002. Phenolic compounds: A review of their possible roles as *in vivo* antioxidants of wine. S. Afr. J. Enol. Vitic. 23, 48-61.

De Ley, J., Gillis, M. & Swings, J., 1984. Genus I Acetobacteceae. In: Bergey's Manual of Systematic Bacteriology, vol. 1 (9^{th}) ed. Williams & Wilkens, Maryland, U.S.A.

Delfini, C., Gaia, P., Schellino, R., Strano, M., Pagliara, A. & Ambrò, S., 2002. Fermentability of grape must after inhibition with dimethyl dicarbonate (DMDC). J. Agric. Food Chem. 50, 5605-5611.

Delves-Broughton, J., 1990. Nisin and its uses as food preservative. Food Technol. 44, 100-117.

Dicks, L.M.T., Dellaglio, F. & Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig] gen.nov., comb. nov. Int. J. Syst. Bacteriol. 45, 395-397.

Drysdale, G.S. & Fleet, G.H., 1985. Acetic acid bacteria in some Australian wines. Food Technol. Aust. 37, 17-20.

Drysdale, G.S. & Fleet, G.H., 1988. Acetic acid bacteria in winemaking: A review. Am. J. Enol. Vitic. 39, 143-154.

Drysdale, G.S. & Fleet, G.H., 1989. The effect of acetic acid bacteria upon the growth and metabolism of yeast during the fermentation of grape juice. J. Appl. Bacteriol. 67, 471-481.

Du Toit, W.J., 2000. Sources of acetic and other fatty acids and their role in sluggish and stuck red wine fermentation. Masters thesis, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa.

Du Toit, M. & Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - A review. S. Afr. J. Enol. Vitic. 21, Special Issue, pp. 74-96.

Du Toit, W.J. & Lambrechts, M.G., 2002. The enumeration and identification of acetic acid bacteria from South African red wine fermentations. Int. J. Food Microbiol. 74, 57-64.

Du Toit, W.J. & Pretorius, I.S., 2002. The occurrence, control and esotreric effect of acetic acid bacteria in winemaking. Ann. Microbiol. 52, 155-179.

Du Toit, W.J., Pretorius, I.S. & Lonvaud-Funel, A., 2005. The effect of sulpur dioxide and oxygen on the viabillty and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. J. Appl. Microbiol. 98, 862-871.

Düring, K., Porsch, P., Mahn, A., Brinkmann, O. & Gieffers, W., 1999. The non-enzymatic microbiocidal activity of lysozyme. FEBS Lett. 449, 93-100.

Dykes, G.A., 1995. Bacteriocins: ecological and evolutionary significance. Tree 10, 186-189.

Edwards, C.G. & Beelman, R.B., 1987. Inhibition of the malolactic bacterium, *Leuconostoc oenos* (PSU-1), by decanoic acid and subsequent removal of the inhibition by yeast ghosts. Am. J. Enol. Vitic. 38, 239-242.

Edwards, C.G., Jensen, K.A., Spayd, S.E. & Seymor, B.J., 1991. Isolation and characterization of native strains of *Leuconostoc oenos* from Washington state wines. Am. J. Enol. Vitic. 42, 219-225.

Edwards, C.G. & Jensen, K.A., 1992. Occurrence and characterization of lactic acid bacteria from washington state wines: *Pediococcus* spp. Am. J. Enol. Vitic. 43, 233-238.

Edwards, C.G., Powers, J.R., Jensen, K.A., Weller, K.M. & Peterson, J.C., 1993. *Lactobacillus* spp. from washington state wines: isolation and characterization. J. Food Sci. 58, 453-458.

Edwards, C.G., Haag, K.M., Collins, M.D., Hutson, R.A. & Huang, Y.C., 1998a. *Lactobacillus kunkeei* sp. nov.: a spoilage organism associated with grape juice fermentations. J. Appl. Microbiol. 84, 698-702.

Edwards, C.G., Haag, K.M. & Collins, M.D., 1998b. Identification and characterization of two lactic acid bacteria associated with sluggish/stuck fermentations. Am. J. Enol. Vitic. 49, 445-448.

Edwards, C.G., Haag, K.M., Semon, M.J., Rodriguez, A.V., Mills, J.M., 1999. Evaluation of processing methods to control the growth of *Lacobacillus kunkeei*, a micro-organism implicated in sluggish alcoholic fermentations of grape musts. S. Afr. J. Enol. Vitic. 20, 11-18.

Edwards, C.G., Collins, M.D., Lwason, P.A. & Rodriguez, A.V., 2000. *Lactobacillus nagelii* sp. nov., an organism isolated from partially fermented wine. Int. J. Syst. Evol. Microbiol. 50, 699-702.

Farías, M.E. & Manca de Nadra, M.C., 2000. Purification and partial characterization of *Oenococcus oeni* exoprotease. FEMS Microbiol. Lett. 185, 263-266.

Ferrando, M., Güell, C. & López, F., 1998. Industrial wine making: comparison of must clarfication treatments. J. Agric. Food Chem. 46, 1523-1528.

Fleet, G.H., 1998. The microbiology of alcoholic beverages. In: Wood, B.J.B. (ed). *Microbiology of Fermented Foods*, vol. 1. Blackie Academic & Professional, London. pp. 217-262.

Fugelsang, C.C., Johansen, C., Christgau, S. & Adler-Nissen, J., 1995. Antimicrobial enzymes: applications and future potential in food industry. Trends Food Sci. Tech. 6, 390-396.

Gänzle, M.G., Weber, S. & Hammes, W.P., 1999. Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. Int. J. Food Microbiol. 46, 207-217.

Garcia-Cazorla, J. & Xirau-Vayreda, M., 1994. Persistence of dicarboximidic fungicide residues in grapes, must, and wine. Am. J. Enol. Vitic. 45, 338-340.

Gao, Y-C., Zhang, G., Krentz, S., Darius, S., Power, J. & Lagarde, G., 2002. Inhibition of spoilage lactic acid bacteria by lysozyme during wine alcoholic fermentation. Aust. J. Grape Wine Res. 8, 76-83.

Gerbaux, V., Villa, A., Monamy, C. & Bertrand, A., 1997. Use of lysozyme to inhibit malolactic fermentation and to stabilize wine after malolactic fermentation. Am. J. Enol. Vitic. 48, 49-54.

Gerbaux, V., Meistermann, E., Cottereau, P.H., Barrère, C., Cuinier, C., Berger, J.L. & Villa, A., 2000. Use of lysozyme in enology. 2nd Int. Viticulture & Oenology Congress. 8-10 Nov. 2000, Cape Town.

Gergely, S., Bekassy-Molnar, E. & Vatai, Gy., 2003. The use of multiobjective optimization to improve wine filtration. J. Food Eng. 58, 31-316.

Gerland, C. & Vialatte, M. Use of lysozyme in enology: applications and limits. Martin Vialette Oenologie - Station Oenotechnique de Champagne.

Gibson, Q.H., Swoboda, B.E.P. & Massey, V., 1964. Kinetics and mechanism of action of glucose oxidase. J. Biol. Chem. 239, 3927-3934.

German, J.B. & Walzem, R.L., 2000. The health benefits of wine. Annu. Rev. Nutr. 20, 561-593.

Grimaldi, A., Mclean, H. & Jiranek, V., 2000. Identification and partial characterization of glycosidic activities of commercial strains of the lactic acid bacterium, *Oenococcus oeni*. Am. J. Enol. Vitic. 51, 362-369.

Guerra, N.P., Luisa Rua, M. & Pastrana, L., 2001. Nutritional factors affecting the production of two bacteriocins from lactic acid bacteria on whey. Int. J. Food Microbiol. 70, 267-281.

Guerra, N.P. & Pastrana, L., 2002. Modelling the influence of pH on the kinetics of both nisin and pediocin production and characterization of their functional properties. Process Biochem. 37, 1005-1015.

Guerrini, S., Mangani, S., Granchi, L. & Vincenzini, M., 2001. Biogenic amine production by *Oenococcus oeni*. Curr. Microbiol., 44, 374-378

Guerrini, S., Bastianini, A., Granchil, L. & Vincenzini, M., 2002. Effect of oleic acid on *Oenococcus oeni* strains and malolactic fermentation in wine. Curr. Microbiol. 44, 5-9.

Guerzoni, M.E., Sinigaglia, M., Gardini, F., Ferruzzi, M. & Torriani, S., 1995. Effects of pH, temperature, ethanol, and malate concentration on *Lactobacillus plantarum* and *Leuconostoc oenos*: modelling of the malolactic activity. Am. J. Enol. Vitic. 46, 368-374.

Guzzo, J., Jobin, M-P. & Diviès, C., 1998. Increase of sulfite tolerance in *Oenococcus oeni* by means of acidic adaptation. FEMS Microbiol. Lett. 160, 43-47.

Héchard, Y. & Sahl, H., 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 84, 545-557.

Helander, I.M., von Wright, A. & Matilla-Sandholm, T-M., 1997. Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. Trend. Food Sci. & Techn. 8, 146-150.

Henick-Kling, T., Acree, T. & Edinger, W., 1998. Sensory impact of malolactic fermentation. Intervitis, 11-17 May, Stuttgart, Germany, pp. 193-197.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T., 1994. Genus *Acetobacter* and *Gluconobacter*. In: Bergey's Manual of Determinative Bacteriology. 9th (ed.), Williams & Wilkens, Maryland, U.S.A.

Hughey, V.L. & Johnson, E.A., 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. Appl. Environ. Microbiol. 53, 2165-2170.

Hughey, V.L., Wilger, P.A. & Johnson, E.A., 1989. Antibacterial activity of hen egg white lysozyme against *Listeria monocytogenes* Scott A in foods. Appl. Environ. Micrbiol. 55, 631-638.

FORDRAS S.A. Influence of technological aids on lysozyme activity in the enology field.

JianFen, L. & Yong, L., 2004. Effect of natamycin on growth of yeast. J. China Agric. Uni. 9, 96-99.

Joyeux, A., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984a. Evolution of acetic acid bacteria during fermentation and storage of wine. Appl. Environ. Microbiol. 48, 153-156.

Joyeux, A., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984b. Metabolism of acetic acid bacteria in grape must: consequences on alcoholic and malolactic fermentation. Sci. Aliments 4, 247-255.

Keilin, D. & Hartree, E.F., 1948. Specificity of glucose oxidase (notatin). Biochem. J. 50, 331-341.

King, S.W. & Beelman, R.B., 1986. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. Am. J. Enol. Vitic. 37, 53-60.

Konings, W.L., Kok, J., Kuipers, O.P. & Poolman, B., 2000. Lactic acid bacteria: the bugs of the new millenium. Curr. Opinion Microbiol. 3, 276-282.

Lafon-Lafourcade, S. Geneix, C. & Ribéreau-Gayon, P., 1984. Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by yeast ghosts. Appl. Environ. Microbiol. 47, 1246-1249.

Leroy, F., Degeest, B. & De Vuyst, L., 2002. A novel area predictive modelling: describing the functionality of beneficial microorganisms in foods. Int. J. Food Microbiol. 73, 251-259.

Liu, J-W.R. & Gallander, J.F., 1983. Effect of pH and sulphur dioxide on the rate of malolactic fermentation in red table wines. Am. J. Enol. Vitic. 34, 44-46.

Liu, S.-Q. & Pilone, G.J., 1998. Arginine metabolism in wine lactic acid bacteria and its pracrical significance: A review. J. Appl. Microbiol. 84, 315-327.

Lonvaud-Funel, A., Joyeux, A. & Desens, C., 1988. Inhibition of malolactic fermentation of wines by products of yeast metabolism. J. Sci. Food Agric. 44, 185-191.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek 76, 317-331.

Lourens, K. & Reid, G., 2002. Yeast nutrient management in winemaking. The Australian & New Zealand Grapegrower & Winemaker: Annual Technical Issue, 50-54.

FORDRAS S.A. Lysozyme in wine production.

Maicas, S., Gil, J., Pardo, I. & Ferrer, S., 1999. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food. Res. Int. 32, 491-496.

Malherbe, D.F., 2002. Expression of the *Apergillus niger* glucose oxidase gene in *Saccharomyces cerivisiae*. Masters thesis, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa.

Malherbe, D.F., Du Toit, M., Cordero Otero, R.R., Van Rensburg, P. & Pretorius, I.S., 2003. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. Appl. Microbiol. Biotechnol. 61, 502-511.

Marchal, R., Chaboche, D., Marchal-Delahaut, L., Gerland, C., Gandon, J.P. & Jeandet, P., 2000. Detection and quantification of lysozyme in Champagne wines. J. Agric. Food Chem. 48, 3225-3231.

Marchal, R., Chaboche, D., Douillard, R. & Jeandet, P., 2002. Influence of lysozyme treatments on Champagne wine foaming properties. J. Agric. Food Chem. 50, 1420-1428.

McKenzie, H.A. & White, F.H., 1991. Lysozyme and α-lactalbumin: structure, function, and interrelationships. Adv. Prot. Chem. 41, 173-315.

McLeod, R. & Ough, C.S., 1970. Some recent studies with glucose oxidase in wine. Am. J. Enol. Vitic. 21, 54-60.

Mira de Orduña, R.M., Liu, S.-Q., Patchett, M.L. & Pilone, G.J., 2000. Ethyl carbamate precursor citrulline formation from arginine degradation by malolactic wine lactic acid bacteria. FEMS Microbiol. Lett. 183, 31-35.

Mira de Orduña, R.M., Patchett, M.L., Liu, S.-Q. & Pilone, G.J., 2001. Growth and arginine metabolism of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. Appl. Environ. Microbiol. 67, 1657-1662.

Nielsen, J.C., Prahl, C. & Lonvaud-Funel, A., 1996. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. Am. J. Enol. Vitic. 47, 42-48.

Nielsen, J.C. & Richelieu, M., 1998. Control of flavor development in wine during and after malolactic fermentation by *Oenococcus oeni*. Appl. Environ. Microbiol. 65, 740-745.

Ohno, N. & Morrison, D.C., 1989. Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme activity. J. Biol. Chem. 264, 4434-4441.

Osborne, J.P., Mira de Orduña, R., Pilone, G.J. & Liu, S.–Q., 2000. Acetaldehyde metabolism by wine lactic acid bacteria. FEMS Microbiol. Lett. 191, 51-55.

O'Sullivan, L., Ross, R.P. & Hill, C., 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84, 593-604.

Ough, C.S., 1975a. Further investigations with glucose oxidase-catalase enzyme systems for use with wine. Am. J. Enol. Vitic. 26, 30-36.

Ough, C.S., 1975b. Dimethyldicarbonate as a wine sterilant. Am. J. Enol. Vitic. 26, 130-133.

Ough, C.S., Kunkee, R.E., Vilas, M.R., Bordeu, E. & Huang, M.-C., 1988. The interaction of sulphur dioxide, pH, and dimethyl dicarbonate on the growth of *Saccharomyces cerevisiae* Montrachet and *Leuconostoc oenos* MCW. Am. J. Enol. Vitic. 39, 279-282.

Palacois, V.M., Caro, I. & Pérez, L., 2002. Comparative study of crossflow microfiltration with conventional filtration of sherry wines. J. Food Eng. 54, 95-102.

Peterson, T.W. & Ough, C.S., 1979. Dimethyldicarbonate reaction with higher alcohols. Am. J. Enol. Vitic. 30, 119-123.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1998. Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. Food Res. Int. 31, 685-692.

Pickering, G.J., Heatherbell, D.A. & Barnes, M.F., 1999. The production of reduced-alcohol wine using glucose oxidase treated juice. Part II. Stability and SO_2-binding. Am. J. Enol. Vitic. 50, 299-306.

Pilatte, E., Naigaard, M., Gao, Y., Krentz, S., Power, J. & Lagarde, G., 2000. Study of the effect of lysozyme on different strains of *Oenococcus oeni*. Applications in the management of malolactic fermentation. Originally published in French in Revenue Française d'Oenologie.

Porter, L.J. & Ough, C.S., 1982. The effects of ethanol, temperature, and dimethyl dicarbonate on viability of *Saccharomyces cerevisiae* Montrachet no. 522 in wine. Am. J. Enol. Vitic. 33, 222-225.

Power, J. 1998. Enzymes in brewing. In Enzymes in Food Processing, 455-456. Edited by T. Nagodawithana & G. Reed. New York, NY: Academic Press, Inc.

Pretorius, I.S., Van der Westhuizen, T.J. & Augustyn, O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. S. Afr. J. Enol. Vitic. 20, 61-74.

Proctor, V.A. & Cunningham, F.E., 1988. The chemistry of lysozyme and its use as food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. 24, 359-395.

Radler, F., 1990a. Possible use of nisin in winemaking. I. Action of nisin against lactic acid bacteria and wine yeasts in solid and liquid media. Am. J. Enol. Vitic. 41, 7-11.

Radler, F., 1990b. Possible use of nisin in winemaking. II. Experiments to control lactic acid bacteria in the production of wine. Am. J. Enol. Vitic. 41, 1-6.

Rankine, B.C. & Bridson, D.A., 1971. Bacterial spoilage in dry red wine and its relationship to malo-lactic fermentation. Aust. Wine & Brewing & Spirit Rev. 0, 44, 46, 48, 50.

Reguant, C., Bordons, A., Arola, L. & Rozès, N., 2000. Influence of phenolic compounds on the physiology of *Oenococcus oeni* from wine. J. Appl. Microbiol. 88, 1065-1071.

Ribéreau-Gayon, P., 1985. New developments in wine microbiology. Am. J. Enol. Vitic. 39, 1-10.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000a. The Microbiology of Wine and Vinifications. Handbook of Enology, vol. 1, John Wiley & Sons Ltd.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000b. The Chemistry of Wine and stabilization and Treatments. Handbook of Enology, vol. 2, John Wiley & Sons Ltd.

Rice-Evans, C.A., Miller, N.J. & Paganda, G., 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Rad. Res.* 22, 375-383.

Rice-Evans, C.A., Miller, N.J. & Paganda, G., 1997. Antioxidant properties of phenolic compounds. *Trend Plant Sci. Rev.* 2, 152-159.

Riley, M.A. & Gordon, D.M., 1999. The ecological role of bacteriocins in bacterial competition. Trends in Microbiol. 7, 129-133.

Riley, M.A. & Wertz, J.E., 2002. Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie 84, 357-364.

Rozès, L., Arola, L. & Bordons, A., 2003. Effect of phenolic compounds on the co-metabolism of citric acid and sugars by *Oenococcus oeni* from wine. Lett. Appl. Microbiol. 36, 337-341.

Ruiz, A., Poblet, M., Mas A. & Guillamon, J.M., 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int. J. Syst. Microbiol., 50, 1981-1987.

Russel, A.D., Hugo, W.B. & Ayliffe, G.A.J., 1991. Chemical food preservatives. In: Priciples and practice of disinfection, preservation and sterilization, 4th edition, Blackwell Scientific Publications, Oxford, London.

Saguir, F.M. & Manca de Nadra, M.C., 2002. Effect of L-malic and citric acids metabolism on the essential amino acid requirements for *Oenococcus oeni* growth. J. Appl. Microbiol. 93, 295-301.

Salton, M.R.J., 1957. The properties of lysozyme and its action on microorganisims. Bacteriol. Rev. 21, 82-99.

Salton, M.R. & Pavilik, J.G., 1960. Studies of the bacterial cell wall. VI. Wall composition and sensitivity to lysozyme. Biochim. Biophys. Acta 39, 398.

Salou, P., Leroy, M.J., Goma, G. & Pareilleux, A., 1991. Influence of pH and malate-glucose ratio on the growth kinetics of *Leuconostoc oenos*. Appl. Microbiol. Biotechnol. 36, 87-91.

Sauvageot, F. & Vivier, P., 1997. Effects of malolactic fermentation on sensory properties of four Burgundy wines. Am. J. Enol. Vitic. 48, 187-192.

Shauz, F., 1996. Centrifuges used in wine clarifaction. Aust. Grapegrower & Winemaker, 392, 42-43.

Schillinger, U. & Holzapfel, W.H., 1996. Guidelines for manuscripts on bacteriocins of lactic acid bacteria. Int. J. Food Microbiol. 33, iii-v.

Somers, T.C. & Wescombe, L.G., 1982. Red wine quality: the critical role of SO_2 during vinification and conservation. Aust. Grapegrower & Winemaker 220, 68, 70, 72, 74.

Sophianopoulos, A.J. & Van Holde, K.E., 1964. Physical studies of murimidase (lysozyme). J. Biol. Chem. 239, 2516-2524.

Stafford, P.A. & Ough, C.S., 1976. Formation of methanol and ethyl methyl carbonate by dimethyl dicarbonate in wine and model solutions. Am. J. Enol. Vitic. 27, 7-11.

Stead, D., 1993. The effect of hydroxynnamic acids on the growth of wine-spoilage lactic acid bacteria. J. Appl. Bact. 75, 135-141.

Stead, D., 1994. The effect of chlorogenic, gallic and quinic acids on the growth of spoilage strains of *Lactobacillus collinoides* and *Lactobacillus brevis*. Lett. App. Micobiol. 18, 112-114.

Strasser de Saad, A.M. & Manca de Nadra, M.C., 1992. Sugar and malic acid utilization and acetic acid formation by *Leuconostoc oenos*. World J. Microbiol. Biotech. 8, 280-283.

Stratiotis, A.L. & Dicks, L.M.T., 2002. Identification of *Lactobacillus* spp. isolated from different phases during the production of a South African fortified wine. S. Afr. J. Enol. Vitic. 23, 14-21.

Tchelistcheff, A., Peterson, R.G. & Van Gelderen, M., 1971. Control of malo-lactic fermentation in wine. Am. J. Enol. Vitic. 22 1-5.

Tegmo-Larsson, I.-M., Spittler, T.D. & Rodriguez, S.B., 1989. Effect of malolactic fermentation on ethyl carbamate formation in Chardonnay wine. Am. J. Enol. Vitic. 40, 106-108.

Temple, D. & Ough, C.S., 1975. Inhibition of catalase activity in wines. Am. J. Enol. Vitic. 26, 92-96.

Ubeda, J.F. & Briones, A.I., 1999. Microbiological quality control of filtered and non-filtered wines. Food Cont. 10, 41-45.

Usseglio-Tomasset, L., 1992. Properties and use of sulphur dioxide. Food Addit. Contam. 9, 399-404.

Vally, H. & Thompson, P.J., 2001. Role of sulfite additives in wine induced asthma: single dose and cumulative dose studies. Thorax 56, 763-769.

Van de Wiel, A., Golde, P.H.M. & Hart, H.Ch., 2001. Blessings of the grape – Review article. Eur. J. Int. Med. 12, 484-489.

Vergès, M.C., Zuñiga, M., Morel-Deville, F., Pérez-Martínez, G., Zagorec, M. & Ehrlich, S.D., 1999. Relationship between arginine degradation, pH and survival in *Lactobacillus sakei*. FEMS Microbiol. Lett. 180, 297-304.

Vidal, M.T., Poblet, M., Constantí, M. & Bordons, A., 2001. Inhibitory effect of copper and dichlofluanid on *Oenococcus oeni* and malolactic fermentation. Am. J. Enol. Vitic. 52, 223-229.

Vivas, N., Lonvaud-Funel, A. & Glories, Y., 1997. Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. Food Microbiol. 14, 291-300.

Vivas, N., Augustin, M. & Lonvaud-Funel, A., 2000. Influence of oak wood and grape tannins on the lactic acid bacterium *Oenococcus oeni* (*Leuconostoc oenos*, 8413). J. Sci. Food Agric. 80, 1675-1678.

Wasserfall, F. & Teuber, M., 1979. Action of egg white lysozyme on *Clostridium tyrobutyricum*. Appl. Environ. Microbiol. 38, 197-199.

Walker, G.M. 1998. Yeast: Physiology and Biotechnology. John Wiley & Sons Ltd, England.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. & Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine. A rHeview. Am. J. Enol. Vitic. 36, 302-313.

Wibowo, D., Fleet, G.H., Lee, T.H. & Eschenbruch, R.E., 1988. Factors affecting the induction of malolactic fermentation in red wines with *Leuconostoc oenos*. J. Appl. Bact. 64, 421-428.

Wilkinson, B.R. & Dorrington, E.E., 1975. Lysozyme (murimidase) from waste egg white. Process Biochem. 10, 24.

Xie, Y.T., Chen, P. & Wei, W.Z., 1999. Rapid analysis of preservatives in beverages by ion chromatography with series piezoelectic quartz crystal as detector. Microchem. J. 61, 58-68.

Yamada, Y., Hoshino, K. & Ishikawa, T., 1997. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: The elevation of the subgenus *Gluconobacter* to the generic level. Biosci. Biotech. Biochem., 61, 1244-1251.

Yoo, W. & Rand, A.G., 1995. Antibacterial effect of glucose oxidase on growth of *Pseudomonas fragi* as related to pH. J. Food Sci. 60, 868-879.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nurry, F.S., 1995. Wine Analysis and Production. Chapman & Hall, New York.

http://www.lysozyme.com

http://www.primepharma.co.za

http://www.sawis.co.za

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CHAPTER 3

RESEARCH RESULTS

The evaluation of lysozyme under winemaking conditions

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3. RESEARCH RESULTS

THE EVALUATION OF LYSOZYME UNDER WINEMAKING CONDITIONS

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Abstract

Lysozyme is an enzyme, commercially extracted from hen-egg white and regularly used in foods. This enzyme exerts bacteriolytic activity towards all Gram-positive organisms. Lysozyme was also approved for usage in wine by the OIV. Thus, lysozyme (EC 3.2.1.17) was evaluated under South African winemaking conditions. Firstly, the influence of lysozyme on different strains of lactic acid bacteria (LAB) and the effect on the alcoholic fermentation tempo (AFT) using small-scale fermentations was investigated. Secondly, the effect of lysozyme on acetic acid bacteria (AAB) and LAB numbers during Pinotage, Cabernet Sauvignon and Shiraz red wine vinifications were also elucidated. Other parameters monitored were volatile components, biogenic amine levels, colour and total phenol content.

It was shown that lysozyme was effective in lowering, or completely inhibiting, LAB growth with the exception of a few strains, thus indicating differences in sensitivity towards lysozyme between species and strains. No effect was observed for AFT. Furthermore, lysozyme treatment did not result in an increase in AAB growth during alcoholic fermentation (AF), however, in some cases resulted in lower AAB numbers for lysozyme treated wines. The effect on the volatile compounds could not be established. Lysozyme treatment did not have any effect on colour or total phenol content of red wine. Biogenic amine content needs further investigation. This is the first evaluation of lysozyme under South African wine conditions.

3.1 INTRODUCTION

Lactic acid bacteria (LAB) are very important in the food and beverage industry. In winemaking, LAB are primarily important for the conversion of the dicarboxylic malic acid into the monocarboxylic lactic acid and carbon dioxide (CO_2), known as malolactic fermentation (MLF) (Lonvaud-Funel, 1995). Genera of LAB associated in winemaking include *Oenococcus*, *Pediococcus*, *Lactobacillus* and *Leuconostoc*, of which *Oenococcus oeni* [formerly known as *Leuconostoc oenos* (Dicks *et al.*, 1995)] is preferred to conduct the MLF (Stiles & Holzapfel, 1997; Lonvaud-Funel, 1999). This conversion is accompanied by changes in pH (increase of 0.2 to 0.5 pH units) and potential organoleptic changes of which diacetyl production are the most important. Several authors described the positive

contributions of LAB in winemaking of which increased complexity and mouth feel were the most prominent organoleptic changes (Henick-Kling *et al.*, 1998; Macais *et al.*, 1999).

Species of the genera of *Lactobacillus* and *Pediococcus* are most often associated with wine spoilage. Several studies have shown the negative aspects associated with spoilage LAB, which include biogenic amines, off-flavours, volatile acidity (VA), ropiness, ethyl carbamate, bitterness (acrolein), mannitol formation, geranium tone and potential sluggish and stuck alcoholic fermentation (Lonvaud-Funel *et al.*, 1988; Huang *et al.*, 1996; Henick-Kling *et al.*, 1998; Lonvaud-Funel, 1999; Maicas *et al.*, 1999; Du Toit & Pretorius, 2000; Guerrine *et al.*, 2001).

Gram-negative bacteria, such as acetic acid bacteria (AAB), are also present during winemaking and are not affected by cell wall degrading enzymes such as lysozyme. The species involved in winemaking are of the genera *Gluconobacter*, *Acetobacter* and *Gluconoacetobacter* (Joyeux *et al.*, 1984a, b; Drysdale & Fleet, 1988; Yamada *et al.*, 1997; Ruiz *et al.*, 2000; Du Toit & Pretorius, 2003). Spoilage of wine by AAB is mainly due to the conversion of either glucose or ethanol to acetic acid. The formation of acetic acid causes an increase of VA. This can be detrimental to wine quality if the concentrations of VA exceed 0.8 g/L. Many countries have imposed strict maximum levels of VA in wine.

One of the primary agents used to control microorganisms in wine is sulphur dioxide (SO_2). SO_2 has long been used for its duel function as an anti-oxidative and antimicrobial agent. SO_2 is active against all microorganisms at lower pH levels (< 3.5). Spoilage strains of LAB, AAB and yeast such as *Brettanomyces* can be controlled effectively by the correct additions of SO_2 (Ribèreau-Gayon *et al.*, 2000b). Excessive concentrations of SO_2 should be avoided for health reasons. These excessive SO_2 concentrations could also interfere with wine bouquet and be a possible source of hydrogen sulphide (H_2S) formation (Gerbaux *et al.*, 1997). Due to the inefficiency of SO_2 at elevated pH values (> 3.5) the need for an additional microbial stabilising agent exists. Gao *et al.* (2000) explained that only the free molecular form exerts antimicrobial activity and only 5-10% SO_2 is present in this form in wines with pH 3 and close to zero in wines with pH 4.

Consumer awareness of chemicals/preservatives in foods and beverages has increased and thus the increasing demand for wines with lowered sulphite levels. Other chemicals' efficiency has also been tested on wine such as sorbic acid, benzoic acid, fumaric acid, natamycin, dimethyl dicarbonate etcetera (Tchelistcheff *et al.*, 1971; Zoecklein *et al.*, 1995; Ribèreau-Gayon *et al.*, 2000b; Delfini *et al.*, 2002; JianFen & Yong, 2004). Sterile filtration and heat treatment can also be used for the microbiological stabilisation of wine but is not absolute. Especially heat treatment can alter the composition of wine components and thus also the organoleptic characteristics of the product. Hence, heat is used more often on winery equipment and barrels than on wine (Zoecklein *et al.*, 1995; Ribèreau-Gayon *et al.*, 2000b).

Lysozyme (EC 3.2.1.17), found in hen egg white and other mammalian fluids, is a naturally occurring bacteriolytic enzyme (Mckenzie & White, 1991). It is officially described as an N-acetylhexosaminodase which consists of 129 amino acids (Wilkinson &

Dorrington, 1975; Mckenzie & White, 1991) and is effective in lysing the cell wall of different bacterial species (Fordras S.A.). It is one of the most popular and safest bactericidal proteins available for commercial use. It has an antimicrobial action only on Gram-positive bacteria (Arima *et al.*, 1997).

Lysozyme provokes a rupture of the β (1-4) linkages between the N-acetylmuramic acid and the N-acetylglucosamine peptidoglycan. The lysing of the peptidoglycan by lysozyme leads to the disruption of the cell walls and cell death due to osmotic shock (Pilatte *et al.*, 2000, McKenzie & White, 1991). Since 2000, lysozyme has been approved in winemaking by the *Office International de la Vigne et du Vin* (OIV). However, certain questions need to be answered in terms of protein stability in white wines, reaction with phenolic compounds and consequently the influence on the colour of the treated wine, especially red wines. The differences between LAB genera and species also need to be addressed in terms of potential resistance towards lysozyme. Whether the elimination of LAB by lysozyme addition can enhance the growth or survival of AAB is not known either.

Thus, the main objective of this study was to investigate different effects of lysozyme usage in winemaking. Firstly, the effect of lysozyme on the survival of different wine-associated LAB type, reverence and wine isolate strains was investigated in laboratory scale experimental Chenin blanc winemaking trials. Subsequently, the effect of lysozyme's on the alcoholic fermentation tempo (AFT) was investigated in these experiments. During the AF the accumulated weight loss was determined. Secondly, the influence of lysozyme on LAB and AAB cell numbers during small scale red wine vinifications was investigated. Subsequently, the colour, phenolic content, biogenic amine content and the volatile components found in wine was also investigated.

3.2 MATERIALS AND METHODS

3.2.1 MEDIA AND REAGENTS

The natural flora of the juice was determined before and after pH adjustments (results not shown) by selecting for LAB strains on De Man, Rogosa and Sharpe (MRS) agar (De Man *et al.*, 1960) and MRS agar (Biolab, Merck, South Africa) enriched with 20% apple juice (containing no preservatives) (MRSA) (pH 5.5). Yeast Peptone Dextrose (YPD) agar (Merck, Biolab Diagnostics (Pty) Ltd) was used for selecting yeast. GYC agar plates (5% glucose, 10% yeast extract, 3% $CaCO_3$, 2% agar) (Drysdale & Fleet, 1988), Modified Carr-agar media [3% yeast extract, 2% agar, 2 mL/L ethanol (96%)] (Gao *et al.*, 2000) and YPM (2.5% mannitol, 0.5% yeast extract, 0.3% peptone, 1.2% agar) were used for the enumeration of AAB.

For the laboratory-scale fermentation enumerations, sample aliquots of 10 µL from a 10-fold dilution series were plated out in triplicate on MRS plates and maintained under facultative anaerobic conditions at 30°C (Anaero-Pack Anaero, Davies Diagnostics (Pty)

Ltd.) in a rectangular anaerobic jar (Davies Diagnostics (Pty) Ltd.) for 7 to 14 days before colonies were enumerated. YPD, GYC, Modified Carr-media and YPM media were aerobically incubated at 30°C until sufficient growth had taken place. During the small-scale red wine fermentations sample aliquots of 100 µL from a 10-fold dilution series were plated out in triplicate on GYC, Modified Carr-media and YPM media.

The MRS, MRSA, GYC, Modified Carr-media and YPM media were supplemented with 100 mg/L Actistab® (50% glucose, 50% natamycin, Gist-brocades, France, S.A., sterilised in 70% ethanol) for the inhibition of moulds, fungi and yeast. Kanamycin sulphate ($C_{18}H_{36}N_4O_{11}$ x H_2SO_4, Roche Diagnostics, dissolved in sterile distilled water) at 25 mg/L were used for the inhibition of AAB in the MRS, MRSA and YPD media and 50 mg/L nisin (Sigma-Aldrich Co., dissolved in methanol) for the inhibition of LAB in the YPD, GYC, YPM and Modified Carr-media respectively. Lysozyme Inovapure 300 (chlorhydrate form, Inovatech, Canada) was used to make a solution of 30 mg/mL by dissolving it in lukewarm sterile distilled water.

3.2.2 LABORATORY-SCALE FERMENTATIONS

Chenin blanc juice was obtained during the 2002 vintage season from Windmeul Cooperative Winery, Wellington, S.A. and stored in 25 L containers at -20°C. This juice was used to investigate the influence of lysozyme on different LAB strains and the AFT. Prior to use the juice was thawed at room temperature and then filtered with a K600 filter membrane and diatomaceous earth (also known as kieselguhr). Analyses of the juice were done by Fourier Transform Infrared Spectrometry (FTIR) (Foss WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss electiric, Denmark, 2001) (**Table 3.1**). Free SO_2 was determined by using the Ripper-method (**Table 3.1**) (Iland *et al.* (1993). Juice pH was adjusted to 3.7 with 1M NaOH and filtered with a 0.45 µm Acetate plus plain filter (Osmonics Inc.). No SO_2 was added to the juice during preparation. Velcorin® (Dimethyl dicarbonate, Bayer AG) was added after filtration of the juice at 0.2 mL/L (diluted in two thirds 96% ethanol prior to addition). The juice was left at room temperature for the Velcorin® to degrade. The wine yeast *Saccharomyces cerevisiae* (VIN 13, Anchor Yeast S.A.) was used for the AF at the recommended standard inoculation dosage of 30 g/hL. The dry yeast was resuspended in a mixture of 70% lukewarm sterile distilled water and 30% sterile juice and allowed to rehydrate for twenty minutes at 37°C. Alcoholic fermentation was conducted at 20±0.5°C. The AFT was monitored daily by determining the accumulated weight loss by weighing the fermentation flasks.

3.2.3 LACTIC ACID BACTERIA STRAINS AND GROWTH CONDITIONS

Five *Lactobacillus* type strains (*L. nagelli* ATCC 700692^T, *L. sakei* subsp. *sakei* LMG 13558^T, *L. pentosus* DSM 20314^T, *L. paracasei* DSM 5622^T *L. buchneri* DSM 20057^T), four reverence strains (*L. plantarum* LMG 13556, *L. fermentum* LMG 13554, *L. vermiforme* NCDO 962 and *Pediococcus acidilactici* PAC 1.0) and seventeen wine isolated LAB

species (*L. paracasei* # 84, *L. plantarum* # 14, *L. pentosus* # 42, *L. brevis* #81.1, *L. paraplantarum* #107.1, *P. acidilactici* #118, *L. plantarum* # 50, *L. paraplantarum* # 101, *L. paracasei* # 54, *L. brevis* #111, *L. brevis* J23, *L. pentosus* K22, *L. buchneri* V1, *L. plantarum* K57, *L. paracasei* L43, *L. hilgardii* M52 and *L. vermiforme* W16) were investigated in this study.

The bacteria were enumerated on MRS from -80°C freeze cultures and incubated anaerobically at 30°C. After sufficient growth on the plates had taken place, a single colony was inoculated into sterile five mL liquid MRS media and incubated at 30°C for ten to twelve hours before inoculating into the grape juice. Inoculation levels were 10^4 to 10^6 colony forming units per millilitre (CFU/mL). After the inoculation of the LAB in grape juice, enumerations were done to determine the inoculation concentration of LAB. The juice and bacteria were simultaneously incubated at 30°C for 48 hours which served as an acclimatisation period for tested LAB species. Samples were taken after the acclimatisation period of 48 hours and an enumeration was done to determine the CFU/mL count at day zero prior to yeast and lysozyme addition.

3.2.4 EXPERIMENTAL DESIGN FOR LABORATORY-SCALE FERMENTATIONS

Two concentrations of lysozyme were used in this experiment for all of the LAB cultures: 0 mg/L, 250 mg/L and 500 mg/L. If a sample showed no CFU/mL for two consecutive enumerations it was considered to be zero and no further enumeration was done for that particular treatment. Lysozyme and yeast were simultaneously added after the acclimatisation period of 48 hours, thus allowing LAB to become established in the juice. Juices were thus sterilised by filtration and Velcorin® addition, left two days, inoculated with the LAB, left for another two days and inoculated with yeast. All lysozyme treatments were carried out in duplicate and enumerations in triplicate. Juice volumes were approximately 150 mL. The AF was conducted in 200 mL clear glass medical round bottles (Laboratory & Scientific Equipment Company, (Cape) (Pty) Ltd.) and fitted with fermentation caps.

3.2.5 SMALL-SCALE RED WINE VINIFICATIONS

Pinotage and Cabernet Sauvignon grapes were obtained from Windmeul Cooperative Winery, Wellington, S.A. in the Paarl wine of origin region. Shiraz grapes were obtained from Vlottenburg Cooperative Winery, Stellenbosch, S.A. in the Stellenbosch wine of origin region. The individual grapes varieties were crushed and destemmed separately and mixed in one container to exclude any variations between crates. Conventional parameters, such as degrees balling (°B), titratable acidity (TA) and pH were determined according to standard procedures (Iland *et al.*, 1993) Values obtained are given in **Table 3.3**.

The TA of the juice was considered to be sufficient and thus no tartaric acid was added to any of the three cultivars. SO_2 was added to the container at a concentration of

20 mg/L by using a 2.5% SO_2 solution to avoid oxidation (calculated at 65% of juice yield/kg grapes) and mixed with a wooden plunger. The container was left overnight at 4°C before the grapes were divided into separate 20L plastic buckets. This was done to minimise spontaneous fermentation by native yeast flora.

Each bucket received 10.8 kg of grapes and juice. All experiments were done in triplicate. The S. *cerevisiae* strain WE372 (Anchor Yeast S.A.) was used for the AF and inoculated at a recommended concentration of 30 g/hL. The dry yeast was resuspended in a mixture of 70% lukewarm sterile distilled water and 30% juice and allowed to rehydrate for 20 minutes at 37°C. Each container received the exact same amount of activated yeast. Lysozyme was added (0 mg/L, 125 mg/L and 250 mg/L) to the grapes (calculated at 65% of juice yield/kg grapes) and mixed. The AF was conducted at room temperature and the °B and temperature of each container were determined each morning and late afternoon by means of a balling meter (results not shown). Each container received three punch downs per day to mix the skins and fermenting juice by using a wooden plunger. The wooden plunger was rinsed between treatments with a mixture of ethanol and distilled water to exclude any recontamination between treatments. Di-ammonium phosphate was added two days after the initiation of AF at a concentration of 0.5 g/L (calculated at 65 % juice yield/kg grapes).

CFU/mL counts of LAB and AAB were done at three stages of the winemaking process to determine the effect of lysozyme: (i) after allocation to the different containers but before yeast inoculation and lysozyme addition (ii) at the middle of fermentation (11-13°B) and (iii) at the end of fermentation prior to the pressing of the grapes (0-1°B). This was done by taking a sample from the middle of the container after punching down.

3.2.6 COLOUR ANALYSES

Iland *et al.* (2000) reported that wines with higher wine colour density, higher total red pigment content and total phenolics are the most full bodied styles and do score well in comparative sensory assessments, provided other wine components are in balance. Spectral measures of wines can thus act as indicator of wine style (and in some cases quality) and are thus a useful analytical tool.

3.2.6.1 Method of colour analyses

Samples were taken of the three different cultivars and treatments at the middle and end of AF. All the samples were clearly marked and stored at -20°C until the measurements were done, thus excluding variations between sampling time and different treatments. Samples for analyses were thawed at room temperature and filtered with a 0.45 µm Acetate plus plain filter (Osmonics Inc). The initial pH values were recorded prior to colour measurements (results not shown).The procedure of colour analyses was done according to Iland *et al.* (2000).

The results of degree of red pigment colouration, estimate of SO_2 resistant pigments, total red pigments, modified wine colour density, modified wine colour hue, modified degree of red pigment colouration and modified estimate of SO_2 resistant pigments are not shown due to no differences.

3.2.7 EXTRACTION OF VOLATILE COMPOUNDS AND CHEMICAL ANALYSIS

The different treatments of each cultivar were divided after AF into two further experiments. The first group received 50 mg/L SO_2 direct after pressing of the grapes, thus preventing MLF and stored at 15°C. The other group underwent MLF by inoculating the wine with a malolactic starter culture Oenoferm Beta (*O. oeni*. Lallemand), 2.5 gram for 2.5 hL wine. The recommended rehydration of the freeze dried bacteria was done in 100 mL distilled water at 20-30°C for 15 minutes. The wine was mixed after inoculation and MLF was carried out at room temperature. Wines were checked regularly for completion of MLF by the FTIR by measuring the malic and lactic acid content. When the wines completed MLF SO_2 was added at a concentration of 50 mg/L and kept at 15°C until samples were taken.

The analyses of the volatile compounds in the different red wines were analysed by gas chromatography (GC) using a Hewlett Packard HP 5890 Gas Chromatograph. The samples for analyses were prepared with a liquid-liquid extraction procedure described by Lilly *et al.* (2000). Alterations of the method included 10 mL of wine as apposed to 50 mL wine used, 800 µL of internal standard added as opposed to 4 mL and ~ 6.5 mL diethyl ether as opposed to 30 mL.

3.2.8 BIOGENIC AMINE CONTENT

Samples of the different red wines were taken after AF to determine if lysozyme had any effect on the biogenic amine content of wine. Two samples of each treatment were filter sterilised by a 0.45 µm Acetate plus plain filter (Osmonics Inc). Analyses of the samples were done by Distell, South Africa by using high-pressure liquid chromatography (HPLC). The method was determined by the modification of Alberto *et al.* (2002). Major changes include the use of different chemicals for the preparation of the derivatising solution, the amount of sample used for derivatisation and the derivatisation reaction time.

3.2.8.1 Instrumentation

All models were from Aligent Technologies compromising the Model HP 1100 series with Chemstation software fitted with the 1100 fluorescence detector set at 340 nm excitation and 420 nm emission wavelengths. The column was a Zobrax SB-C18, 5 µm, 4.6 x 150 mm equipped with a security guard holder plus C18 cartridge, both from Phenomenex.

3.2.8.2 Mobile phase and chromatographic conditions

The same mobile phase, gradient and chromatographic conditions were used as used by Alberto *et al.* (2002).

3.2.8.3 Derivatisation

The derivatisation reagent comprised 200 mg o-phtaldiadehyde (OPA) (Sigma) in 9 mL methanol, 1 mL 0.1M sodium tetra borate (pH 10) and 160 µL 2-mercaptoethanol. Standard slotions of biogenic amines were prepared by dissolving each amine in methanol. Wine samples were diluted 10 times and filtered through a 0.45 µm syringe filter prior to derivatisation and column injection. 25 µL of the diluted wine sample was reacted with 25 µL of derivatising reagent for exactly 45 seconds and 25 µL of this solution was injected immediately thereafter. The derivatisation process was automated by the making use of the autosampler.

3.3 RESULTS

3.3.1 EFFECT OF LYSOZYME ON LACTIC ACID BACTERIA GROWTH AND ALCOHOLIC FERMENTATION TEMPO

The Chenin blanc juice used in the laboratory scale fermentations were analysed by FTIR (**Table 3.1**). The pH of the juice was considered to be too low to favour the growth of spoilage LAB in must, therefore it was adjusted to 3.7. It was considered that the free SO_2 found by the Ripper-method was not enough to inhibit LAB growth, especially after the pH adjustment, resulting in a diminished fraction of molecular SO_2. The natural flora of the must was determined at pH 3.4 and 3.7 (results not shown).

Table 3.1 Analyses of Chenin blanc juice analysed by FTIR and free SO_2 by the Ripper-method.

pH	Glucose-Fructose (g/L)	Total acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	VA (g/L)	Free SO_2 (mg/L)
3.4	209 g/L	4.9	1.9 g/L	3.4	0.12	10

Of the tested type strains, only the control of *L. nagelii* ATCC 700692^T showed positive growth during the AF with CFU/mL increasing from 10^5 CFU/mL at day zero to almost 10^8 CFU/mL at day nine. In contrast, the treated lysozyme samples showed complete inhibition of LAB at day nine, indicating a 10^8 difference in CFU/mL between the control and lysozyme treated samples. No difference was observed for the two different tested lysozyme concentrations (**Fig. 3.1**). Edwards *et al.* (2000)

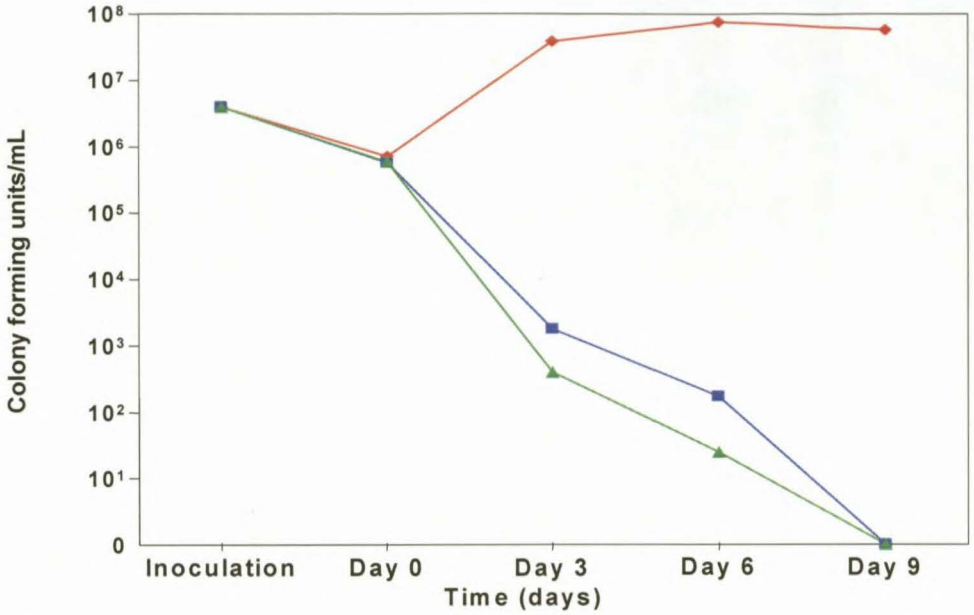


Figure 3.1 The effect of lysozyme on the growth of *Lactobacillus nagelii* ATCC 700692^T. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

identified *L. nagelii* from partially fermented wine, thus indicating that *L. nagelii* might have a higher ethanol resistance than other studied LAB bacteria. No diverse effect on the AFT was found with lysozyme addition (**Fig. 3.2 appendix**). However, it would seem that 500 mg/L and 250 mg/L lysozyme improved the AF with a slightly higher accumulated CO_2 loss during the AF than the control, but these differences were small.

No differences were observed for *L. plantarum* LMG 13556 in CFU/mL numbers, in the case of the control, also decreasing from 10^5 CFU/mL (day zero) to zero cell counts in six days. A 10^2 CFU/mL difference was observed at day three during the AF between the control and the lysozyme treated samples (**Fig. 3.3 appendix**). Krieling (2003) and Du Plessis *et al.* (2004) also found that *L. plantarum* dominates in grape juice. Krieling (2003) however found the predominant species of LAB isolated from grapes and wine was *L. plantarum* and *Pediococcus* spp. Du Plessis *et al.* (2004) reported that the viability *L. plantarum* decreased significantly during the AF of brandy base wines. It would therefore be difficult to conclude that only lysozyme was responsible for the reduction of cell numbers during the AF. The occurrence of these LAB bacteria could also differ between seasons as Krieling (2003) and Du Plessis *et al.* (2004) noted. No differences were found between the control and lysozyme treated wines in terms of the AFT although the 500 mg/L lysozyme had a slighter higher accumulated CO_2 loss (**Fig. 3.4 appendix**).

A slight resistance towards lysozyme treatment was observed for *L. sakei* subsp. *sakei* LMG 13558^T with CFU/mL numbers at day nine dropping to 10^2 -10^3 (lysozyme treated). No difference was observed between the two different tested lysozyme concentrations. The control sample showed positive growth from day zero to day three. However, a difference of 10^2 CFU/mL between the control and lysozyme treated samples was apparent at day nine (**Fig. 3.5 appendix**), thus showing sensitivity to lysozyme treatment.

A higher accumulated CO_2 loss was found in the case of the control and 250 mg/L lysozyme. 500 mg/L lysozyme showed a lower accumulated CO_2 loss during the AF (**Fig. 3.6 appendix**).

L. fermentum LMG 13554's CFU/mL numbers diminished during the six days of AF from 10^5 to zero CFU/mL (control). No increase in numbers where found during the 48 hour acclimatisation period and could indicate that the bacteria only survived in the grape juice, however, relatively high numbers were started with when compared to lower CFU/mL numbers present in grape juice. Lysozyme treated samples showed complete inhibition with a zero CFU/mL count at day three. At day three a 10^4 CFU/mL difference was observed between the control and lysozyme treated samples. No difference was observed between the two different lysozyme concentrations (**Fig. 3.7 appendix**). The growth and survival of *L. fermentum* showed the same tendency as that of *L. plantarum* LMG 13556, thus indicating that the bacteria might prefer an alcohol free environment. It would therefore be inconclusive to say that lysozyme was solely responsible for the reduction in LAB numbers during the AF. No difference was observed between the samples during the AFT (**Fig. 3.8 appendix**).

The control sample of *L. pentosus* DSM 20314^T (**Fig. 3.9**) slightly increased during the first three days of AF with a 10^3 CFU/mL difference between the control and lysozyme treated samples at this stage. The control sample's CFU/mL decreased to 10^3 CFU/mL at day six and a further reduction was observed at day nine with a recorded 10^2 CFU/mL. Lysozyme treated samples showed a sharp decrease in numbers during the AF, especially 500 mg/L lysozyme addition resulting in complete inhibition of LAB at day six, with 250 mg/L addition of lysozyme causing complete inhibition at day nine. A 10^4 CFU/mL difference was observed between 500 mg/L lysozyme and the control values at day six and a 10^2 CFU/mL difference between 250 mg/L lysozyme and the control at day nine.

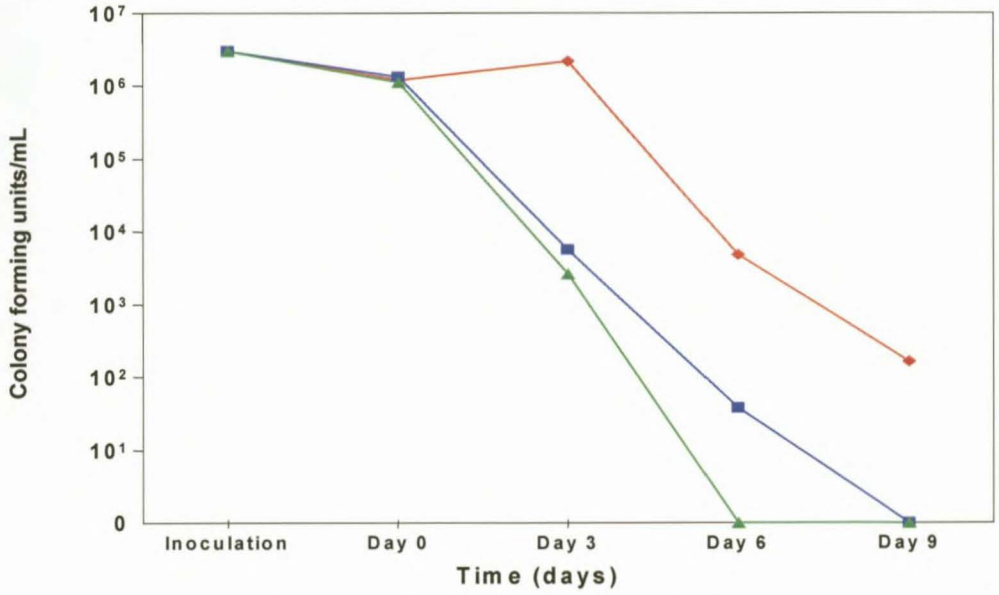


Figure 3.9 The effect of lysozyme on the growth of *Lactobacillus pentosus* DSM 20314^T. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

The decrease in the control's numbers could be due to ethanol sensitivity. No difference in accumulated CO_2 loss was observed between the control and 250 mg/L lysozyme. Five hundred mg/L lysozyme showed a slighter slower accumulated CO_2 loss over time (**Fig. 3.10 appendix**).

Similar trends were observed in the case of *L. paracasei* DSM 5622^T (**Fig. 3.11 appendix**) during the AF, except no net growth was observed for the control during the tested period. However, the control showed a sharp decrease from 10^5 CFU/mL at day zero to 10^3 CFU/mL at day three, but continued to survive until day twelve, with CFU/mL numbers remaining constant between 10^2 and 10^3 CFU/mL. Krieling (2003) and Du Plessis *et al.* (2004) also found *L. paracasei* in table wines and brandy base wine samples respectively, however the occurrence they observed were small. Du Plessis *et al.* (2004) also noted *L. paracasei* numbers developing after MLF, thus indicating that they can survive in a normal wine medium such as brandy base wine. These results correspond to that of Krieling (2003) who also isolated *L. paracasei* from wine and during MLF. The survival of *L. paracasei* could, however, be strain specific.

No differences were found at day three for the two lysozyme concentrations. Five hundred mg/L lysozyme resulted in complete inhibition at day six with zero CFU/mL being recorded at this stage. A 10^3 and 10^1 CFU/mL difference was observed between the control, 500 mg/L lysozyme and 250 mg/L respectively at day six. 250 mg/L lysozyme resulted in complete inhibition at day twelve, with a 10^2 CFU/mL difference when compared with the control. No difference was found between the different treatments of lysozyme in terms of accumulated CO_2 loss (**Fig. 3.12 appendix**).

L. buchneri DSM 20057^T (**Fig. 3.13**) also showed positive results regarding the action of lysozyme with the control CFU/mL numbers dropping slightly from inoculation to day zero and again from day zero to day three. For the control, a sharp decrease was observed between day three and day six with an almost 10^2 difference in CFU/mL numbers. From day six the values for the control stayed relatively constant. Lysozyme addition caused a marked difference in growth when compared to the control. Both lysozyme concentrations resulted in complete inhibition at day three. It could be speculated that inhibition was achieved in a shorter time than three days. A 10^4 CFU/mL difference between the lysozyme treatments and the control was observed at day three. The accumulated CO_2 loss indicated that there was no difference observed between the control and the two lysozyme concentrations during the AF; thus indicating a similar AFT for all tested samples (**Fig. 3.14 appendix**).

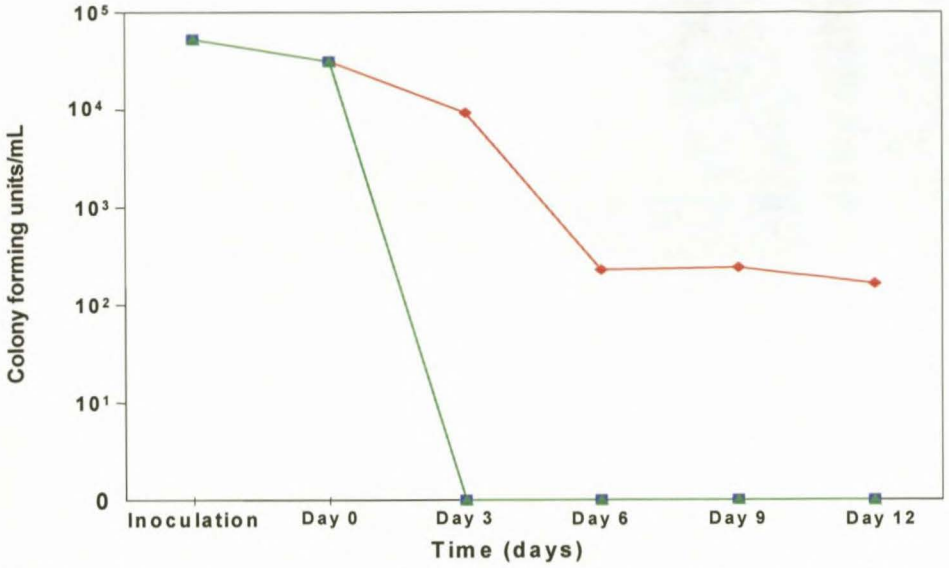


Figure 3.13 The effect of lysozyme on the growth of *Lactobacillus buchneri* DSM 20057^T culture. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

P. acidilactici PAC 1.0 seemed sensitive to the growth conditions in must/grape juice, which can be seen in **Fig. 3.15 appendix**. The control illustrated no growth with CFU/mL numbers dropping from 10^5 to 10^3 during the first three days of AF with a further reduction in numbers to 10^1 at day six. No CFU/mL was recorded at day nine. The two lysozyme concentrations gave a similar result with complete inhibition of *P. acidilactici* PAC 1.0 at day three. It is also possible that complete inhibition was achieved in less than three days of AF. A 10^3 CFU/mL count difference was observed at day three between the control and lysozyme treated samples.

Edwards & Jensen (1992) isolated mainly *P. parvulus* from a 12% ethanol wine and in addition, Krieling (2003) found that the predominant LAB species isolated from grapes and wine (end of alcoholic fermentation and during MLF) in three different cultivars was *L. plantarum* and *Pediococcus* spp. However, Du Plessis *et al.* (2004) did not isolate one *P. acidilactici* strain in three years of analysing brandy base wines, indicating that these LAB species' numbers are possibly limited in juice destined for brandy base wine or are very strain and species specific. Gao *et al.* (2002) found a reduction in cell numbers of *P. damnosus* and *P. parvulus* when treated with 125 mg/L and 250 mg/L lysozyme. The particular vintage could also influence the numbers found in juice and wine. The accumulated CO_2 loss indicated that there was also no difference observed between the control and the two lysozyme concentrations during the AF; thus indicating a similar AFT for all tested samples (**Fig. 3.16 appendix**).

L. vermiforme NCDO 962 (**Fig. 3.17**) showed relative good survival during the initial stages of AF with CFU/mL numbers dropping only from 10^5 CFU/mL to 10^3 CFU/mL during the duration of the experiment. No difference between the two lysozyme concentrations was observed, with both resulting in complete inhibition at day three of AF. Stratiotis &

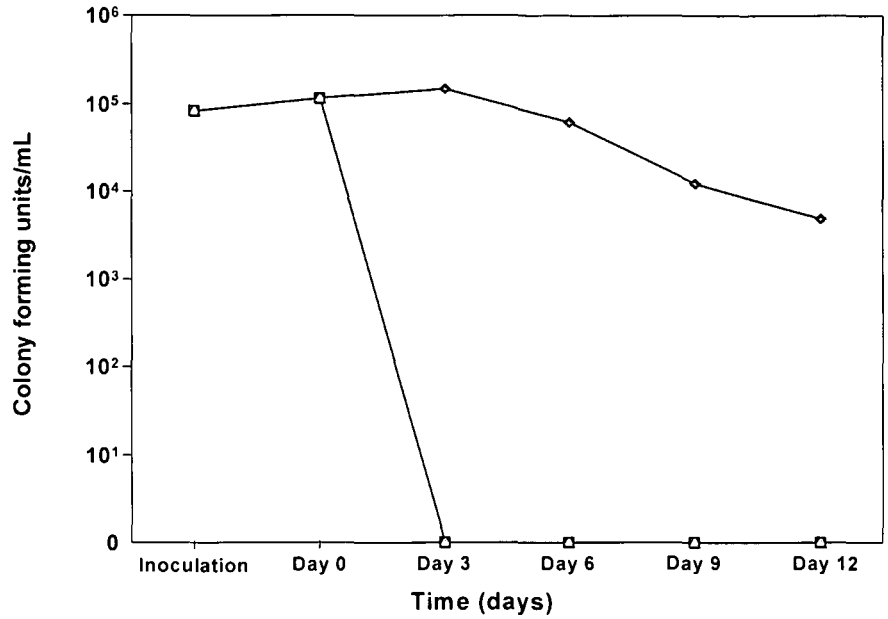


Figure 3.17 The effect of lysozyme on the growth of *Lactobacillus vermiforme* NCDO 962. ◊ Control; □ 250 mg/L lysozyme; Δ 500 mg/L lysozyme.

Dicks (2002) identified *L. vermiforme* strains from South African fortified wines, thus indicating that these LAB species can survive in high ethanol content as is found in fortified wines (> 16% by volume) and could thus be able to survive and grow during a normal alcoholic fermentation. Du Plessis *et al.* (2004) also isolated *L. vermiforme* during the AF of brandy base wine and after MLF were completed. The use of lysozyme in fortified and natural wines could thus inhibit these LAB species, however, the sensitivity to lysozyme could also be strain specific. A 10^5 CFU/mL difference was observed between the control and the lysozyme treated wines at day three of AF and 10^4 CFU/mL for the remainder of the AF until day twelve, thus confirming sensitivity to the tested lysozyme concentrations. **Fig. 3.18** shows that there were no differences in the AFT between the tested samples although 500 mg/L and 250 mg/L lysozyme differed slightly from the control over the tested period.

L. paracasei # 84 CFU/mL numbers increased during the first three days of AF and decreased after day three during the AF for all the samples (**Fig. 3.19 appendix**). The control sample's CFU/mL decreased from day three and onwards, eventually dropping from 10^9 to 10^3 CFU/mL over a twelve day period. The lysozyme treated samples' numbers were from day zero lower than the control sample values. Two hundred and fifty mg/L lysozyme also showed a slight increase in numbers during the first three days of AF;however a sharp decrease in numbers was observed at day six when CFU/mL numbers dropped from 10^8 to 10^6 CFU/mL and again dropping to 10^4 CFU/mL at day nine. The reduction in numbers from day nine until day fifteen differed in 10^2 CFU/mL fractions, thus ending at 10^2 CFU/mL at day fifteen. Five hundred mg/L lysozyme showed similar effects, however ending with 10^1 CFU/mL at day fifteen. The main difference between the tested *L. paracasei* DSM 5622^T (**Fig. 3.11 appendix**) and the locally isolated strain # 84 is

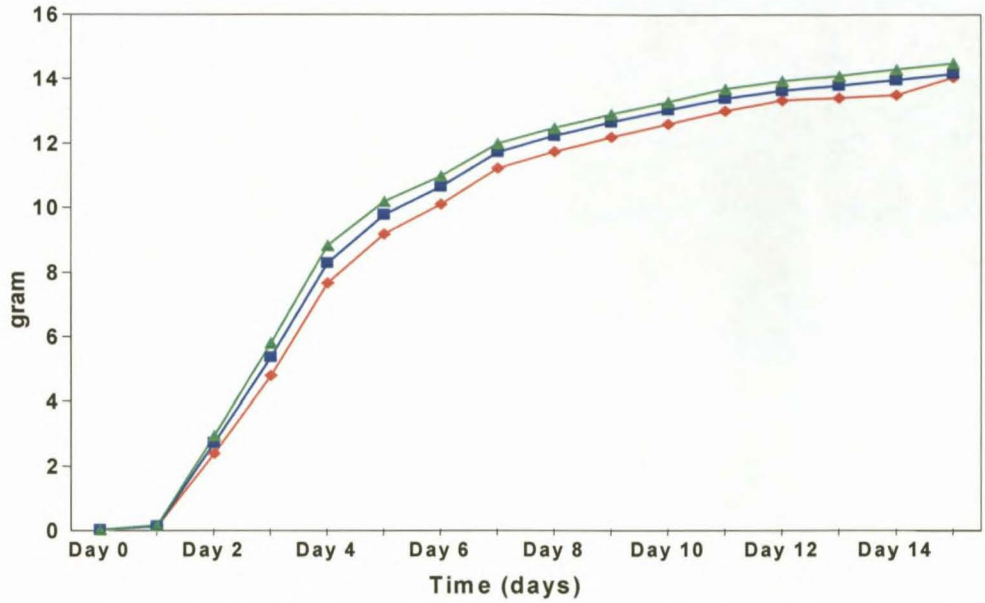


Figure 3.18 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus vermiforme* NCDO 962 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

that the control survived throughout the AF for the latter strain. Krieling (2003) found *L. paracasei* in wine and during MLF. However, the same tendency during the AF (decreasing numbers) was observed. It can be argued that the locally isolated strain was more adapted to local conditions than the type strain. No difference was observed for the AFT between the tested samples (**Fig. 3.20 appendix**).

Similar trends were observed for *L. plantarum* # 14 with growth in the first three days of AF for all the treated samples. Inoculation levels were between 10^5 and 10^6 CFU/mL and all samples showed positive growth during the acclimatisation period of 48 hours. At day zero the recorded CFU/mL counts were 10^8-10^9 CFU/mL, which is extremely high compared to cell numbers found in normal winemaking circumstances. However, a reduction was also observed for the control after three days of AF, which continued in similar decreasing numbers until day fifteen. Lysozyme addition resulted in reduced numbers from day three until day fifteen of AF. No difference was observed between the two different lysozyme concentrations in terms of CFU/mL numbers. A reduction of 10^2, 10^2, 10^1 and 10^1 CFU/mL were observed for days six, nine, twelve and fifteen respectively. A difference of 10^2 CFU/mL was observed at days nine, twelve and fifteen between the control and lysozyme treated wines (**Fig. 3.21 appendix**). It would have been interesting to elucidate the cell numbers at day eighteen of AF. However, it would seem that the wine isolate was more adapted to wine conditions than the tested type strain (**Fig. 3.3 appendix**). Du Plessis *et al.* (2004) found that *L. plantarum* dominates in grape juice and that their viability decreased significantly during the AF of brandy base wines. Krieling (2003) found *L. plantarum* to predominate on grapes and in wine. No difference was observed for the AFT between the tested samples (**Fig. 3.22 appendix**).

(2003) found *L. plantarum* to predominate on grapes and in wine. No difference was observed for the AFT between the tested samples (**Fig. 3.22 appendix**).

The conditions in must during the AF did not support the growth of *L. pentosus* # 42. This can be seen at the hand of a total reduction in numbers from inoculation to day twelve of AF (control). Inoculation levels were between 10^4 and 10^5 CFU/mL. The control's CFU/mL counts were reduced from 10^3-10^4 CFU/mL at day three to 10^1-10^2 CFU/mL at day six, stabilised between days six to nine and were reduced again to zero at day twelve. The most significant effect was seen with 500 mg/L lysozyme addition. Only three, possibly even fewer days were needed for the total inhibition of growth. A zero CFU/mL count was recorded at day three of AF. A difference of 10^3-10^4 CFU/mL between 500 mg/L lysozyme, control and 250 mg/L lysozyme was recorded at day three. Two hundred and fifty mg/L lysozyme resulted in complete inhibition at day six, with a 10^1-10^2 CFU/mL difference between the control and 250 mg/L lysozyme. A nine and six day period of complete inhibition difference was recorded between the control, 500 mg/L and 250 mg/L respectively (**Fig. 3.23 appendix**). The growth of the tested wine isolate during AF correlates to the type strain DSM 20314^T (**Fig. 3.9**) where inhibition was achieved after three days (500 mg/L lysozyme) and six days (250 mg/L lysozyme) respectively, while the control also showed a decrease in numbers during the AF. The 250 mg/L lysozyme treatment and the control recorded an almost identical AFT with the 500 mg/L lysozyme treatment showing a slight improved AFT (**Fig. 3.24 appendix**).

Similar trends were observed with *P. acidilactici* # 118 during the AF as with the tested reverence strain *P. acidilactici* PAC 1.0 (**Fig. 3.15 appendix**). Inoculation levels were 10^5-10^6 CFU/mL and no growth was observed during the acclimatisation period of 48 hours. Similar growth curves were obtained from the three different lysozyme concentrations. No major difference was seen between day zero and day three. Five hundred mg/L lysozyme caused the most significant effect and complete inhibition was achieved at day six of AF. A 10^3-10^4 CFU/mL and 10^2-10^3 CFU/mL difference was seen between the 500 mg/L and 250 mg/l lysozyme respectively compared to the control at day six. Two hundred and fifty mg/L lysozyme resulted in complete inhibition at day nine. The control showed zero CFU/mL at day twelve (**Fig. 3.25 appendix**). Two hundred and fifty mg/L lysozyme and the control recorded an almost identical AFT with 500 mg/L lysozyme recording a slight improved AFT. However, these differences were not significant (**Fig. 3.26 appendix**).

Resistance to lysozyme was observed when it failed to achieve any significant reduction in numbers for the tested *L. brevis* # 81.1 strain (**Fig. 3.27**). Inoculation levels were between 10^5-10^6 CFU/mL. Extremely good growth was observed during the 48 hours acclimatisation period with CFU/mL numbers escalating to 10^8-10^9 CFU/mL. No difference in CFU/mL numbers were recorded between the three tested lysozyme concentrations during the AF. After fifteen days of AF the CFU/mL counts were 10^5 CFU/mL, 10^5-10^6 CFU/mL and 10^4-10^5 CFU/mL over time for the control, 500 mg/L lysozyme and 250 mg/L lysozyme respectfully. This result is contradictory to that of Gao *et al.* (2002) who found the tested *L. brevis* strain to be sensitive to lysozyme additions of 125 mg/L and 250 mg/L,

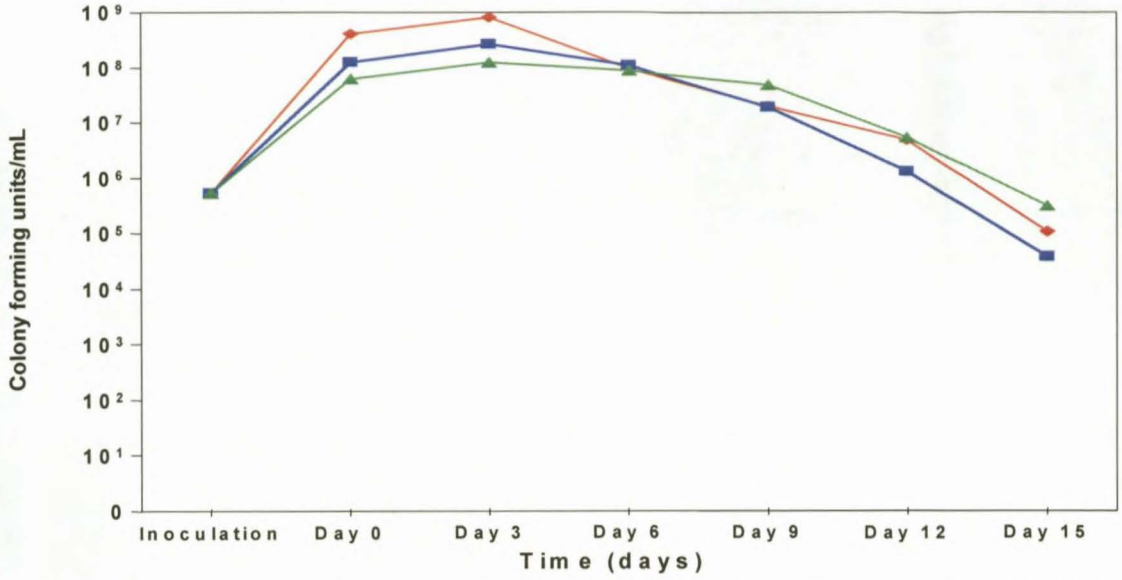


Figure 3.27 The effect of lysozyme on the growth of *Lactobacillus brevis* wine isolate # 81.1. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

what was evaluated in this study. Another difference is that their cell counts continued for 30 days, which in this case was only done up to day fifteen. This could play a significant part in the interpretation of this result, because, in the case of Gao *et al.* (2002), *L. brevis* only showed zero cell counts after 20 plus days when treated with 250 mg/L lysozyme. The AFT also showed some interesting results with the control recording the highest accumulated CO_2 loss during the AF. The lysozyme treated samples recorded an almost identical CO_2 loss during the AF (**Fig. 3.28 appendix**). It thus seems that lysozyme did not inhibit *L. brevis* # 118 over the tested period, but possibly gave the yeast a fermentation advantage.

Relatively good inhibition was observed in the case of *L. plantarum* # 50 when treated with lysozyme. Inoculation levels were 10^4-10^5 CFU/mL. Slightly positive growth was observed in the case of the control and 250 mg/L lysozyme samples during the acclimatisation period. The 500 mg/L lysozyme showed a slight reduction in numbers during this period. The control recorded a positive increase in CFU/mL numbers in the first three days of AF in contrast to the two previously tested *L. plantarum* LMG 13556 and *L. plantarum* # 14 strains, followed by a sharp reduction from 10^5-10^6 CFU/mL to 10^3-10^4 CFU/mL at day six, followed again by an increase from day six to day nine. The 500 mg/L and 250 mg/L lysozyme treatments showed identical growth curves, due the fact that both concentrations resulted in complete inhibition at day six of AF, although 500 mg/L lysozyme resulted in a faster reduction in numbers (**Fig. 3.31 appendix**). It would have been interesting to see how the control's numbers varied until day fifteen to twenty of AF to see whether it corresponds to Du Plessis *et al.* (2004) findings that indigenous *L. plantarum* numbers decreased during the AF in brandy base wines. No difference was observed for the AFT between the tested samples (**Fig. 3.32 appendix**).

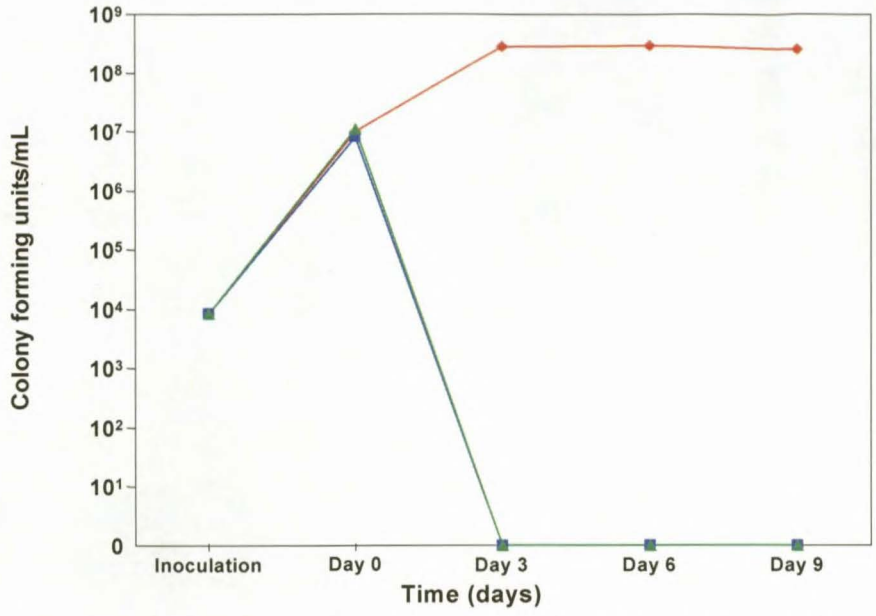


Figure 3.33. The effect of lysozyme on the growth of *Lactobacillus paraplantarum* wine isolate # 101. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

The control sample of *L. paraplantarum* # 101 (**Fig. 3.33**) showed positive growth during the AF. Inoculation levels were 10^4 CFU/mL and numbers increased over the 48 hour acclimatisation period up to 10^7 CFU/mL for all the tested samples. Very good growth was obtained when numbers increased to 10^8-10^9 CFU/mL at day three of AF. The recorded numbers of the control at days six and nine remained relatively constant. Unlike the control 500 mg/L and 250 mg/L lysozyme additions resulted in complete inhibition at day three of AF with zero CFU/mL being recorded at this stage. A difference of 10^8-10^9 CFU/mL was observed between the control and lysozyme treated wines at days three, six and nine of AF. It can be speculated that the inhibition was achieved in less time than three days. No difference was observed for the AFT between the tested samples (**Fig. 3.34 appendix**).

Similar inhibition spectrums were observed when *L. paracasei* # 54 were treated with lysozyme. Inoculation levels were 10^5 CFU/mL. However, no initial growth was observed during the acclimatisation period, with a slight decrease in numbers to 10^4-10^5 CFU/mL at day zero. The control continued a slight decrease in numbers during the first three days of AF after which it increased from day three and continued to increase to 10^6-10^7 CFU/mL at day nine. Du Plessis *et al.* (2004) found *L. paracasei* developing after MLF, thus indicating that it might be more ethanol tolerant than other indigenous LAB. The 250 mg/L and 500 mg/L lysozyme concentrations showed exactly the same result with complete inhibition at day three. A difference of 10^4 CFU/mL was calculated at day three between lysozyme treated and untreated wines (**Fig. 3.35 appendix**). Interesting results were obtained in the case of accumulated CO_2 loss during the AF. No differences between the treatments were obtained from day zero to day four of AF. However, from day five to day fourteen the accumulated CO_2 differed between the samples. The 250 mg/L treatment showed the

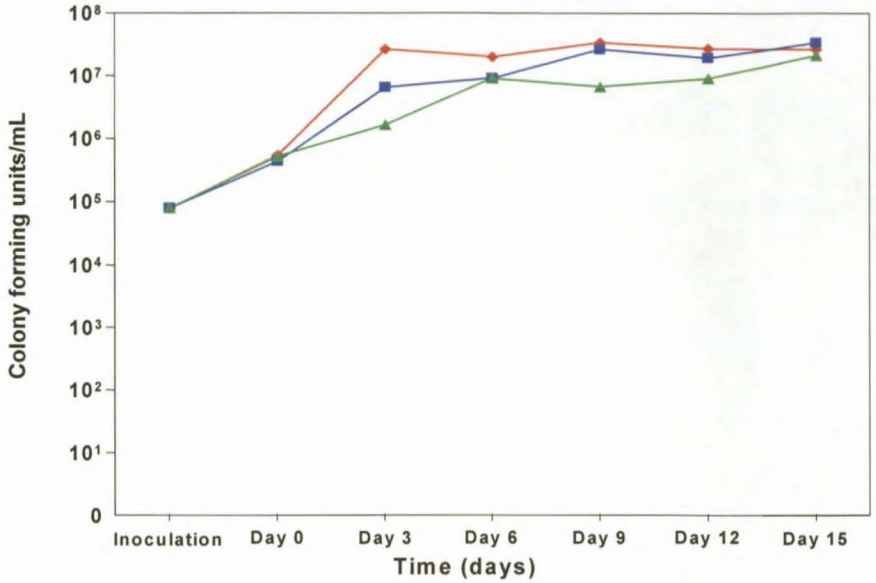


Figure 3.39 The effect of lysozyme on the growth of *Lactobacillus brevis* wine isolate J23. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

highest AFT but no significant difference was found when compared to the 500 mg/L lysozyme treatment. The control sample recorded a slightly lower AFT and lower accumulated CO_2 loss during the AF (**Fig. 3. 36 appendix**).

L. brevis J23 was the second lactobacilli strain to show signs of resistance to lysozyme. This can be seen by results shown in **Fig. 3.39**. Inoculation levels were 10^5 CFU/mL with relatively good growth during the acclimatisation period. All three treatments showed an increase in numbers during the course of the AF, with the control showing the fastest growth during the first three days of AF, which resulted in numbers between 10^7-10^8 CFU/mL. Five hundred mg/L lysozyme did not result in a decrease in numbers, but restricted growth to 10^6 CFU/mL at day three. At the same stage 250 mg/L lysozyme recorded CFU/mL numbers between 10^6 and 10^7. Lysozyme treated samples' CFU/mL numbers continued to be slightly less than those of the control throughout the AF, but eventually reaching the same numbers at day fifteen. This result is again contradictory to that of Gao *et al.* (2002) who found the tested *L. brevis* strain to be sensitive to additions of 125 mg/L and 250 mg/L lysozyme, which is less than what was evaluated in this study. Again the argument arises that their CFU/mL counts continued until day 30 of AF, which in this case was only done up to day fifteen. This could play a significant part in the interpretation of this result, because, in the case of Gao *et al.* (2002), *L. brevis* only showed zero cell counts after 20 plus days when treated with 250 mg/L lysozyme. No difference was observed for the AFT between the tested samples (**Fig. 3.40 appendix**).

L. pentosus K22 showed very good initial growth during the acclimatisation period, when inoculation levels increased from 10^5 CFU/mL to 10^7-10^8 CFU/mL at day zero. The CFU/mL counts for the control seemed to stay constant during the AF, with numbers fluctuating between 10^8-10^9 CFU/mL over this period. Two hundred mg/L and 500 mg/L lysozyme additions resulted in an almost exact decrease in cell numbers at day three. A

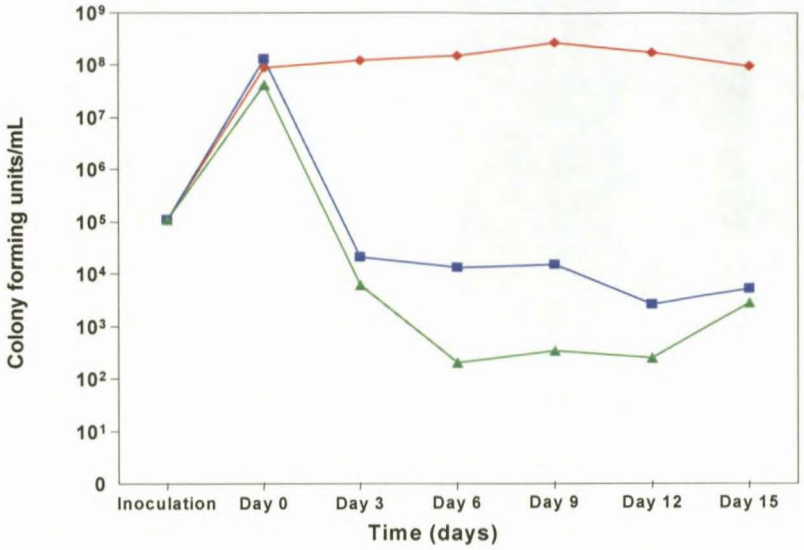


Figure 3.41 The effect of lysozyme on the growth of *Lactobacillus pentosus* wine isolate K22. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

500 mg/L lysozyme treatment continued the decrease in cell numbers until day six when cell numbers of 10^2-10^3 CFU/mL were recorded. However, no total inhibition was achieved for both lysozyme treated wines yet numbers remained constantly lower than the control (10^3 to 10^4 CFU/mL at day fifteen compared to 10^7 to 10^8 CFU/mL for the control). This could be due to the fact that cell counts only continued until day fifteen of AF. Five hundred mg/L lysozyme remained constant from day six onwards and even showed a slight increase at day fifteen. The 250 mg/L lysozyme treatment addition showed relative constant numbers from day three to day nine with a slight decrease in numbers at day twelve. An average difference in cell numbers between the control and 250 mg/L lysozyme during days three to fifteen are in the order of 10^4-10^5 CFU/mL. A 500 mg/L lysozyme addition resulted in an increased difference, if compared to the former concentration, of 10^5-10^6 from days six to fifteen (**Fig. 3.41**). No difference was observed for the AFT between the tested samples (**Fig. 3.42 appendix**).

The third species of *Lactobacillus* to apparently be more resistant to lysozyme is *L. buchneri* V1 (**Fig. 3.43**) in contrast to the tested type strain of *L. buchneri* DSM 20057^T (**Fig. 3.13**). Inoculation levels were 10^5-10^6 CFU/mL. The control sample did not initially respond as good as the other samples. However, good growth was observed for the control during the first three days of AF. The control continued to increase in numbers until day six, when its maximum cell count was reached. From day nine until day fifteen the CFU/mL remained relatively constant. Both lysozyme treated samples also showed good initial growth. Lysozyme addition did not result in inhibition of LAB growth and numbers increased during the course of AF. The fact that the wine isolate is more adapted to wine conditions could one of the reasons for resistance. No difference was observed for the AFT between the tested samples (**Fig. 3.44**).

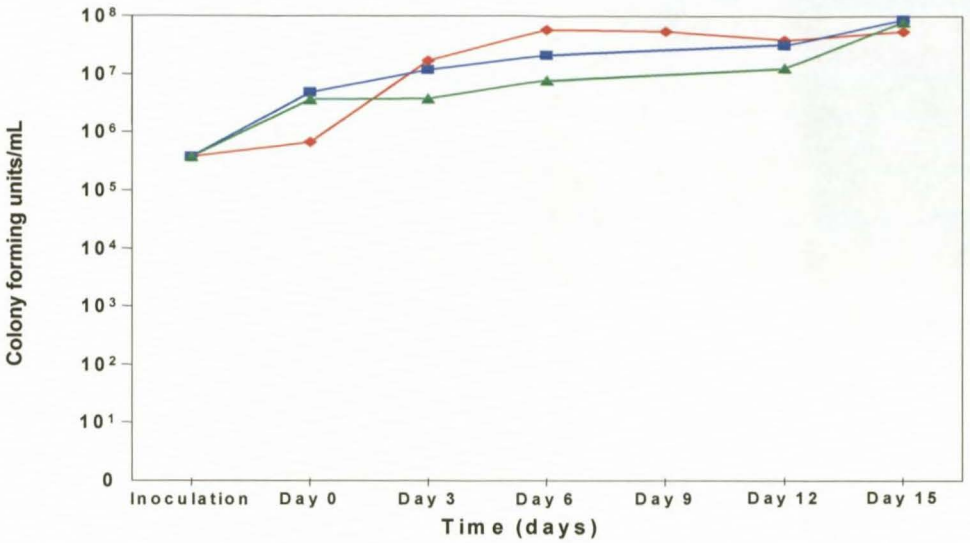


Figure 3.43 The effect of lysozyme on the growth of *Lactobacillus buchneri* wine isolate V1. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

Lysozyme addition, especially 500 mg/L resulted in complete inhibition of *L. plantarum* K57 at day six of AF (**Fig. 3.45**) showing the same tendency as *L. plantarum* # 50 (**Fig. 3.31 appendix**) but differing form *L. plantarum* LMG 13556 and *L. plantarum* # 14. Inoculation levels were 10^5-10^6 CFU/mL and positive growth occurred during the acclimatisation period. The control showed positive growth until day three of AF and continued to remain constant throughout the AF. A 250 mg/L lysozyme addition resulted in a sharp decline in CFU/mL during the first three days of AF. From day three to day twelve there was no major difference in CFU/mL numbers for 250 mg/L lysozyme. A 500 mg/L lysozyme addition resulted in a reduction

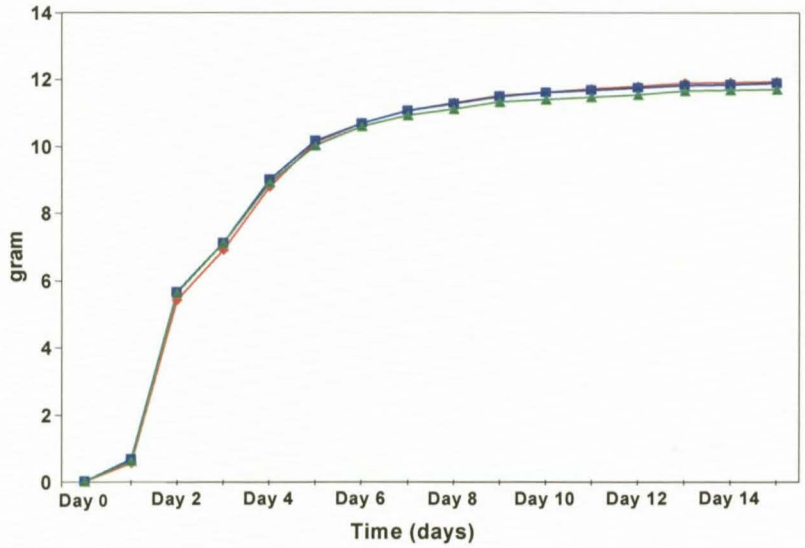


Figure 3.44 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus buchneri* wine isolate V1 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

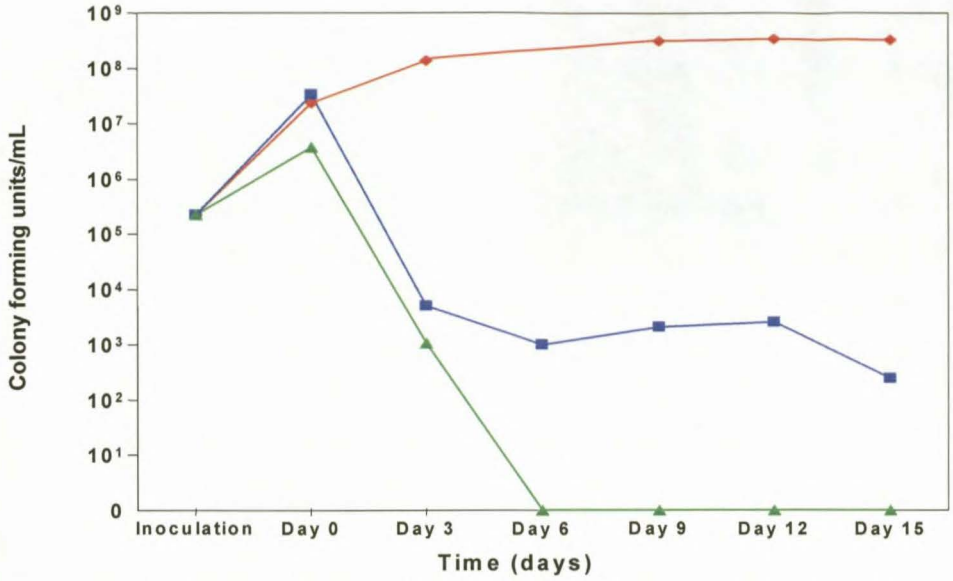


Figure 3.45 The effect of lysozyme on the growth of *Lactobacillus plantarum* wine isolate K57. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

of cell numbers from day zero (10^6-10^7) to 10^3 CFU/mL at day three, with complete inhibition at day six. A 10^5 CFU/mL difference between the control and 500 mg/L lysozyme was observed at day three, followed by a 10^8 CFU/mL difference at day six. This difference remained the same throughout the AF. A 10^3 CFU/mL difference between 250 mg/L and 500 mg/L lysozyme was observed at days six, nine and twelve respectively with a 10^2 difference at day fifteen. A difference of 10^5-10^6 CFU/mL was calculated between the control and 250 mg/L lysozyme from days six to fifteen. It can be speculated that 250 mg/L lysozyme would eventually also decrease to zero numbers however cell counts were only performed until day 15 of AF. No significant differences were observed for the AFT between the tested samples (**Fig. 3.46 appendix**).

The fourth *Lactobacillus* strain showing some resistance to lysozyme is *L. paracasei* L43. Inoculation levels were almost 10^4 CFU/mL and the strain adapted well to the conditions in the must. This can be seen by the increase in numbers over the 48 hour acclimatisation period by all samples to 10^4-10^5 CFU/mL for the control and 500 mg/L lysozyme samples. In the 250 mg/L lysozyme samples day zero values increased to 10^5-10^6 CFU/mL. The control showed a positive increase in numbers in three days of AF to 10^7 CFU/mL and continued to remain constant over the tested period. Although the initial lysozyme concentrations of 250 mg/L were higher at day zero, the CFU/mL numbers never exceeded those of the control. Five hundred mg/L lysozyme showed the lowest CFU/mL counts for the experiment, but did not succeed to inhibit growth during any stage of the AF (**Fig. 3.47**). No differences were observed for the AFT between the tested samples (**Fig. 3.48 appendix**).

Interesting results were obtained during the AF of *L. hilgardii* M52 (**Fig 3.49 appendix**) with lysozyme. Inoculation levels were 10^4-10^5 CFU/mL and an increase in cell numbers

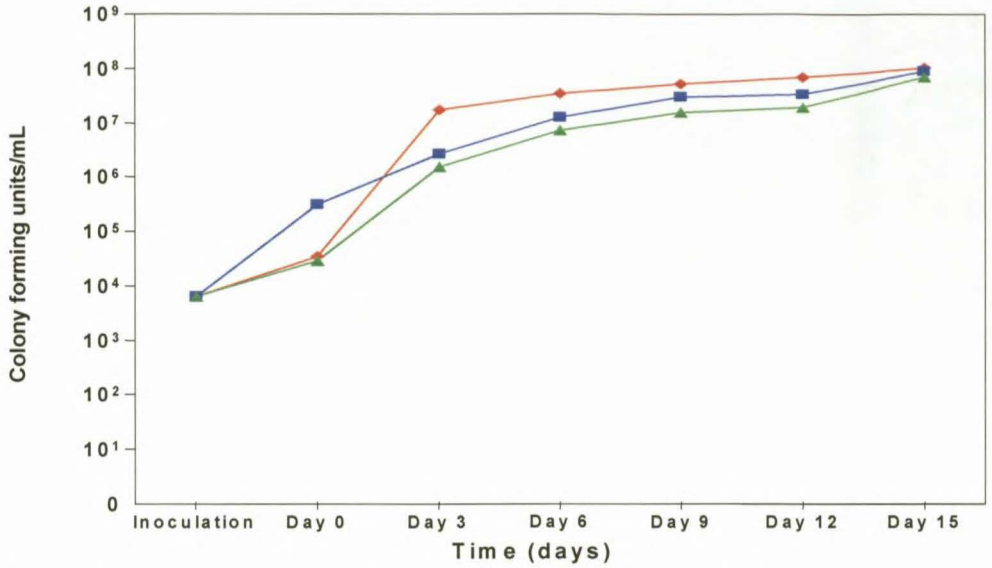


Figure 3.47 The effect of lysozyme on the growth of *Lactobacillus paracasei* wine isolate L43. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

were observed for all samples. The control showed a very good increase in numbers and increased from 10^5-10^6 to 10^7-10^8 CFU/mL in three days of AF. The positive growth continued until day six for the control and remained at constant numbers until the end of AF. This is in contrast to Lafon-Lafourcade *et al.* (1983) who speculated that *L. hilgardii* might be intolerant to alcohol generated during AF. However, Du Plessis *et al.* (2004) found indigenous *L. hilgardii* after MLF in brandy base wines, thus indicating that this species can be found during AF and after MLF. The same reduction in numbers was obtained with 250 mg/L and 500 mg/L lysozyme additions. A reduction of 10^5-10^6 to 10^3 CFU/mL was recorded in the first three days of AF. A 500 mg/L lysozyme addition continued the inhibition of the LAB strain by recording zero CFU/mL at day six of AF. However, one of the experiments showed that growth re-occurred after complete inhibition was achieved at day six by adding lysozyme. Thus the fact that re-contamination may have occurred cannot be excluded and are not further mentioned. A 10^4 difference in CFU/mL counts between the control and 500 mg/L lysozyme were observed at day three with a difference of 10^7 at day six. A cell count difference of 10^2 between 250 mg/L and 500 mg/L were observed at day six. A difference of 10^5 and 10^6 CFU/mL was observed at day nine and day twelve, respectively, between the control and 250 mg/L lysozyme samples. No significant differences were observed for the AFT between the tested samples (**Fig. 3.50 appendix**).

L. vermiforme W16 showed the same sensitivity as the tested reverence strain *L. vermiforme* NCDO 962 (**Fig. 3.17**). Inoculation levels were 10^4 CFU/mL and positive growth was observed during the acclimatisation period of 48 hours with cell numbers increasing to 10^5 CFU/mL. The control continued with positive growth until day three of AF by reaching 10^7 CFU/mL. Cell numbers from day three onwards remained constant for the control sample. In contrast 250 mg/L lysozyme and 500 mg/L lysozyme addition resulted in

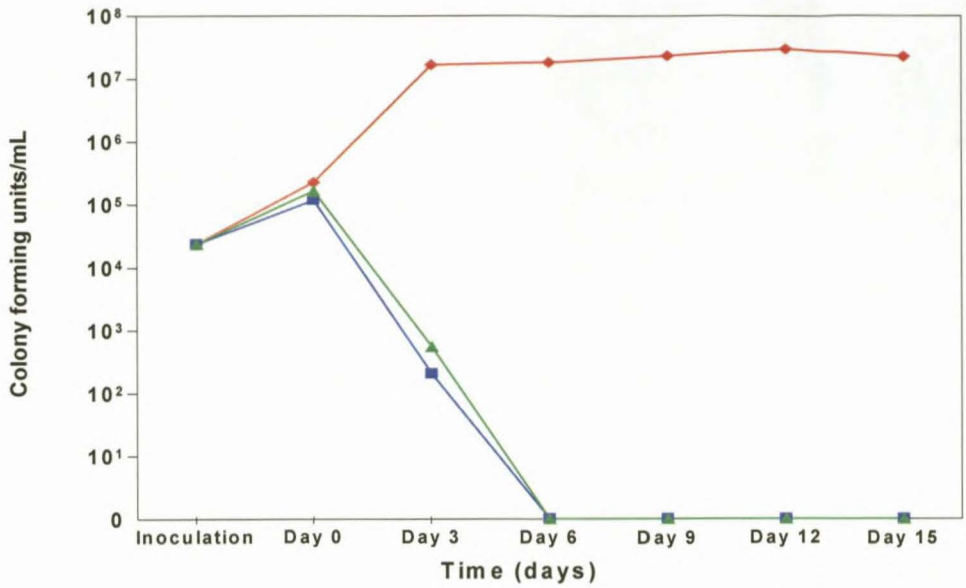


Figure 3.51 The effect of lysozyme on the growth of *Lactobacillus vermiforme* wine isolate W16. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

a 10^3 reduction in cell numbers over a three day period during AF. Both lysozyme additions resulted in complete inhibition of the tested LAB due to zero CFU/mL counts being recorded at day six. A calculated 10^7 CFU/mL difference at day six between the control and two lysozyme concentrations was achieved (**Fig. 3. 51**). Stratiotis & Dicks (2002) identified *L. vermiforme* strains from South African fortified wines, thus indicating that these LAB species can survive in high ethanol content as is found in fortified wines (>16% by volume) and could thus be able to survive and grow during a normal alcoholic fermentation. Du Plessis *et al.* (2004) also isolated *L. vermiforme* during the AF of brandy base wine and after MLF was completed. The use of lysozyme in fortified and natural wines could thus inhibit these LAB species, but the sensitivity to lysozyme could also be strain specific. No differences were observed for the AFT between the tested samples (**Fig. 3.52 appendix**).

The conditions in must did not support the growth of *Leuconostoc mesenteroides* # 5 (**Fig. 3. 53 appendix**) during AF. Inoculation levels were 10^5 CFU/mL with a slight increase during the acclimatisation period. The 250 mg/L and 500 mg/L lysozyme additions resulted in complete inhibition of the tested LAB due to zero CFU/mL recorded at day three. The possibility may exist that inhibition was achieved in less than three days of AF. A 10^3 CFU/mL difference was seen at day three between the control and lysozyme treated samples. The control recorded a zero CFU/mL count at day six of AF. Lafon-Lafourcade *et al.* (1983) reported that *Leuc. mesenteroides* might be sensitive to ethanol production during the AF supporting findings by Du Plessis *et al.* (2004) that found no indigenous *Leuc. mesenteroides* strains in brandy base wines. No differences were found between the control and lysozyme treated wines (results not shown).

3.3.2 THE INFLUENCE OF LYSOZYME ON LAB AND AAB COLONY FORMING UNITS DURING SMALL-SCALE RED WINE VINIFICATIONS

Values of the different parameters measured in the three cultivars used are given in **Table 3.3**. The pH of the Cabernet Sauvignon and Shiraz were relatively high and could have diminished the molecular effect of SO_2. A high pH and °B was recorded for Shiraz. The TA of all the different cultivars was relatively high with Cabernet Sauvignon recording the lowest TA.

Table 3.3 Analysis of Pinotage, Cabernet Sauvignon and Shiraz grapes for physical appearance and chemical composition of juice after crush/destemming of the grapes.

Cultivar	Health status	°B	pH	TA (g/L)
Pinotage	Good, compact bunches	23.8	3.38	6.54
Cabernet Sauvignon	Good, loose bunches	25	3.67	6.30
Shiraz	Good, mushy bunches	26.1	4.06	6.79

The LAB numbers of the three different cultivars differed from each other during the AF. This can clearly be seen on the two different media that was used for the enumeration of LAB. For instance, no difference was observed for LAB numbers in Pinotage must on MRS media (**Fig. 3.54 a**). Day zero CFU/mL numbers revealed that little difference existed between the wines with an average of 8.7×10^2, 1.4×10^3 and 2.2×10^3 for the control, 125 mg/L lysozyme and 250 mg/L lysozyme treatments respectively. Lonvaud-Funel (1995, 1999) also reported that LAB are normally present in low numbers (10^3 CFU/g) on healthy grapes and subsequently on must, thus corresponding to initial numbers found on MRS and MRSA media in Pinotage must. The middle of AF numbers did also not differ between the different treatments with recorded CFU/mL numbers of 9.7×10^2, 8.7×10^2 and 3.8×10^2 CFU/mL for the control, 125 mg/L and 250 mg/L lysozyme treatments respectively. No difference was observed at the end of AF between treatments. There is a possibility that other factors could have influenced this result. For instance, when comparing the medium chain fatty acid content of especially Pinotage and Shiraz after AF, and especially octanoic acid, much higher concentrations of this fatty acid was recorded in Pinotage than Shiraz. Lonvaud-Funel *et al.* (1988) showed that hexanoic-, octanoic- and decanoic acids were more inhibitory towards a *O. oeni* strain when added in combination than alone. Other authors found that the toxicity of octanoic acid increased when the pH of the medium decreased from 5.4 to 3.0 (Caretté *et al.*, 2002).

No differences in CFU/mL were also observed on MRSA media (**Fig. 3.54 b**). Day zero counts varied from 9.7×10^2 for the control to 1×10^3 and 1.2×10^3 for 125 mg/L lysozyme and 250 mg/L lysozyme respectively. The end of AF recorded numbers of 3.3×10^1, 1.6×10^1 and 1.6×10^1 for the control, 125 mg/L lysozyme and 250 mg/L lysozyme respectively.

AAB numbers during the Pinotage AF indicated differences between the Carr media and GYC media (**Fig. 3.54**). No difference was observed between the lysozyme treated and untreated samples on GYC media (**Fig. 3.54 c**). AAB numbers slightly increased to the middle of AF followed by a slight reduction in numbers for both GYC and Carr media (10^4 to 10^2 CFU/mL reduction) (**Fig. 3.54 d**). No AAB counts were recorded on Carr media at the start of the AF. This is probably due to *Gluconobacter* spp. that dominate at the start of fermentation when the main carbon source is glucose, thus indicating their preference for a sugar-rich environment (Splittstoesser & Churney, 1992). Du Toit & Lambrechts (2002) found that *Gluconobacter oxydans* dominated in fresh must and that their numbers decreased during AF on GYC media. An increase in AAB numbers was recorded at the middle of AF for both GYC and Carr media. A 250 mg/L lysozyme addition resulted in a lower AAB CFU/mL count during the middle and end of AF on Carr media. No difference was observed between the control and 125 mg/l lysozyme during the middle and end of AF on Carr media. A tendency in decreasing AAB numbers towards the end of AF can be seen for both enumeration media as well as for all the treatments. Joyeux *et al.* (1984a) and Du Toit & Lambrechts (2002) showed that AAB are able to survive and even grow during the winemaking process.

Differences in LAB numbers, in contrast to that found in the Pinotage AF were recorded during the Cabernet Sauvignon AF (**Fig. 3.55**). Day zero counts on MRS media showed that no difference in CFU/mL numbers with 6×10^3, 6.9×10^3 and 5.2×10^3 for the control, 125 mg/L lysozyme and 250 mg/L lysozyme respectively (**Fig. 3.55 a**), again corresponding to Lonvaud-Funel (1995, 1999) and Fleet's (1998) findings that LAB numbers of 10^3 CFU/g are found on healthy grapes and in musts. Differences of one log CFU/mL were obtained at the middle of AF with the control having 7.2×10^3 CFU/mL and the 125 mg/L 250 mg/L lysozyme addition reducing numbers to 2.8×10^2 CFU/mL and 3.2×10^2 CFU/mL respectively. The end of AF again showed differences between the control (3.7×10^3 CFU/mL) and the lysozyme treated wines. A two log CFU/mL difference between the control and lysozyme treated wines were recorded (125 mg/L lysozyme (8.3×10^1 CFU/mL) and 250 mg/L lysozyme (1×10^1 CFU/mL)) treatments.

The same tendencies were observed on MRSA media as on MRS media (**Fig. 3.55 b**). Day zero counts did not differ significantly (10^3 CFU/mL). One log differences were observed between the control and lysozyme treated wines during the middle and end of AF. The middle of AF numbers show higher numbers for the control (7.3×10^3 CFU/mL) than 125 mg/L lysozyme (2.5×10^2 CFU/mL) and 250 mg/L lysozyme (1.8×10^2 CFU/mL) treatments. Significant differences can be seen at the end of AF. The control almost remained constant with 5.1×10^3 CFU/mL. A reduction in numbers was observed to 1.4×10^1 CFU/mL and 8.3×10^1 CFU/mL for 125 mg/L lysozyme and 250 mg/L lysozyme respectively.

A different tendency regarding AAB numbers were found during the Cabernet Sauvignon as to that of the Pinotage AF. A decrease in AAB numbers was found during the AF for all the samples on both GYC and Carr media. The addition of 250 mg/L

lysozyme resulted in slightly lower CFU/mL numbers during enumeration at the middle AF on GYC media (**Fig. 3.55 c**), however this was not the case with Carr media **(Fig. 3.55 d)**. In contrast to the Pinotage vinification AAB numbers (10^4 CFU/mL) were found at day zero on Carr media. Du Toit & Lambrechts (2002) found *Acetobacter pasteurianus* dominating in must but certain strains of *G. oxydans* are also able to grow on ethanol. The control and 125 mg/L showed similar CFU/mL counts at the middle of AF on GYC media. No CFU/mL counts were recorded at the end of AF for all the samples on both GYC and Carr media (**Fig. 3.55 c, d**).

The results during the Shiraz AF showed completely contrasting results to those of the Pinotage and the Cabernet Sauvignon AF. No difference in CFU/mL numbers were found at day zero (**Fig. 3.56 a**). The middle of AF showed a reduction in numbers to 7.2×10^3 CFU/mL, 2×10^3 CFU/mL and 1.9×10^3 CFU/mL for the control, 125 mg/L lysozyme and 250 mg/l lysozyme respectively. An increase in cell numbers was observed for all samples at the end AF. Thus, lysozyme did not succeed in inhibiting LAB growth during the AF. An increase in numbers was also observed on the MRSA media at the end of AF (**Fig. 3.56 b**).

Initial higher AAB numbers were recorded at the start of AF (day zero) on GYC media (**Fig. 3.56 c**). However, no differences were found between lysozyme treated and untreated samples on GYC media. No differences were found on Carr media between the lysozyme treated and untreated samples (**Fig. 3.56 d**).

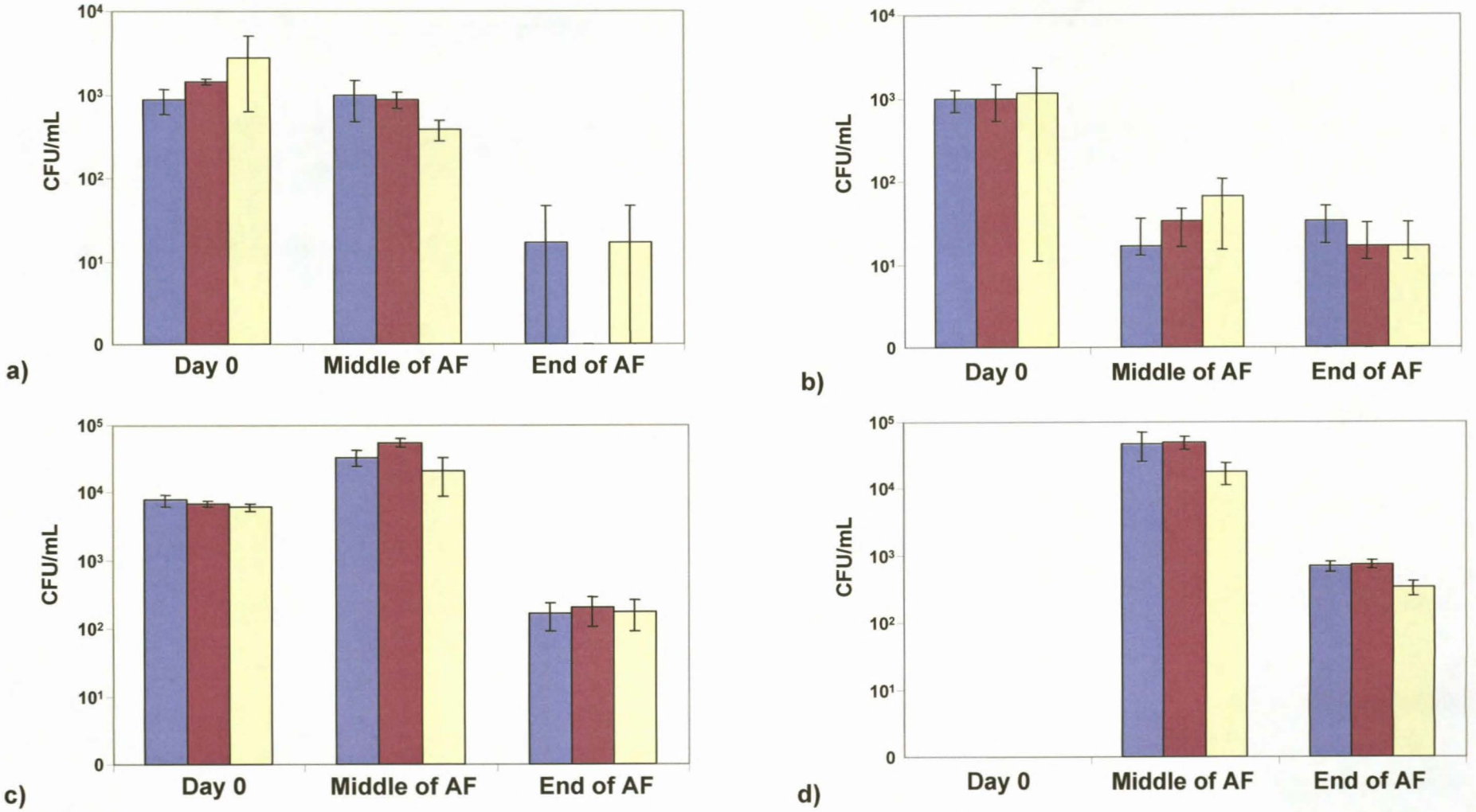


Figure 3.54. The effect of lysozyme on AAB (GYC and CARR media) and LAB (MRS and MRS + apple juice (MRSA)) numbers during small-scale Pinotage vinifications. a) MRS; b) MRSA; c) GYC and d) CARR. ■ Control; ■ 125 mg/L lysozyme; ■ 250 mg/L lysozyme.

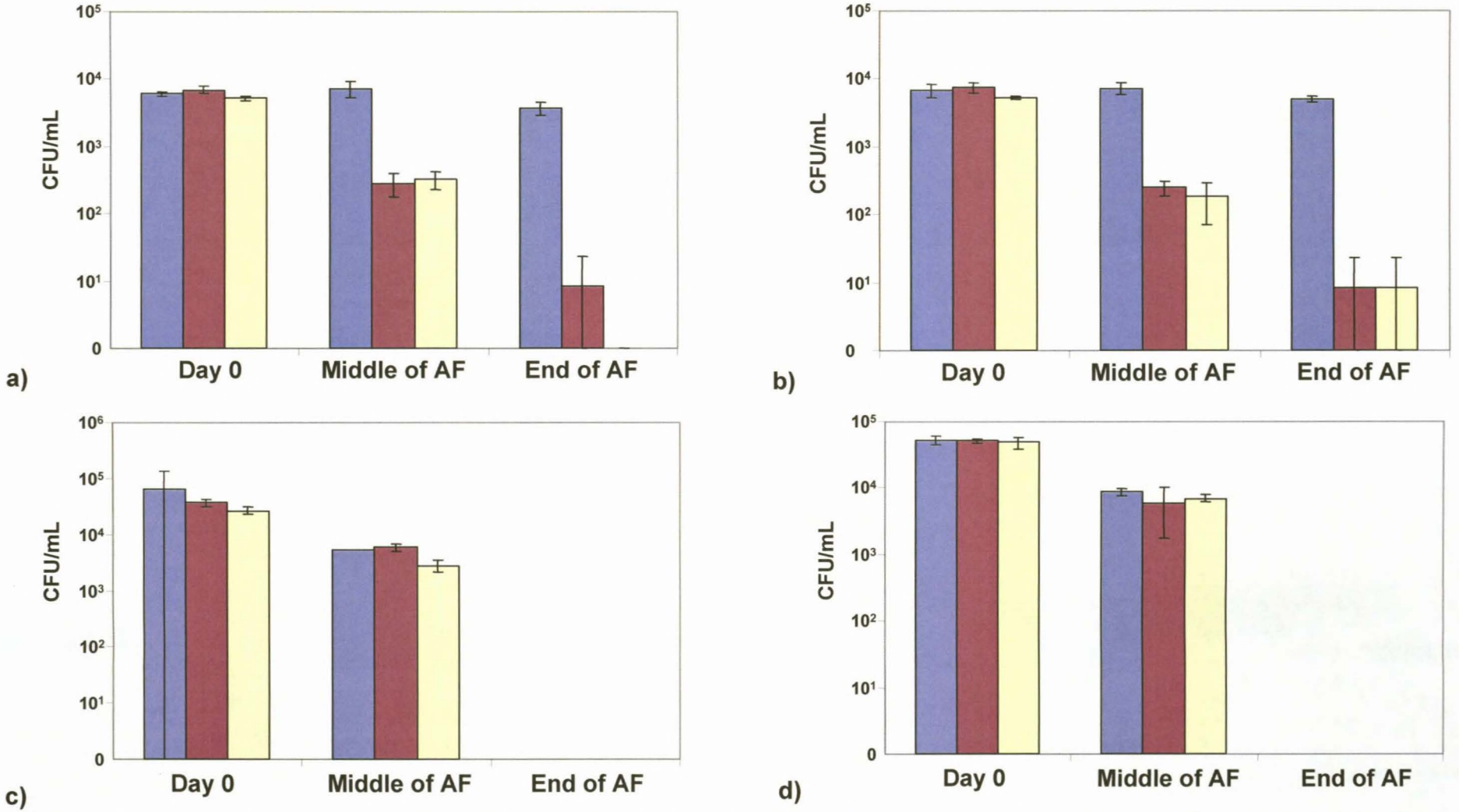


Figure 3.55. The effect of lysozyme on AAB (GYC and CARR media) and LAB (MRS and MRS + apple juice (MRSA)) numbers during small-scale Cabernet Sauvignon vinifications. a) MRS; b) MRSA; c) GYC and d) CARR. ■ Control; ■ 125 mg/L lysozyme; ■ 250 mg/L lysozyme.

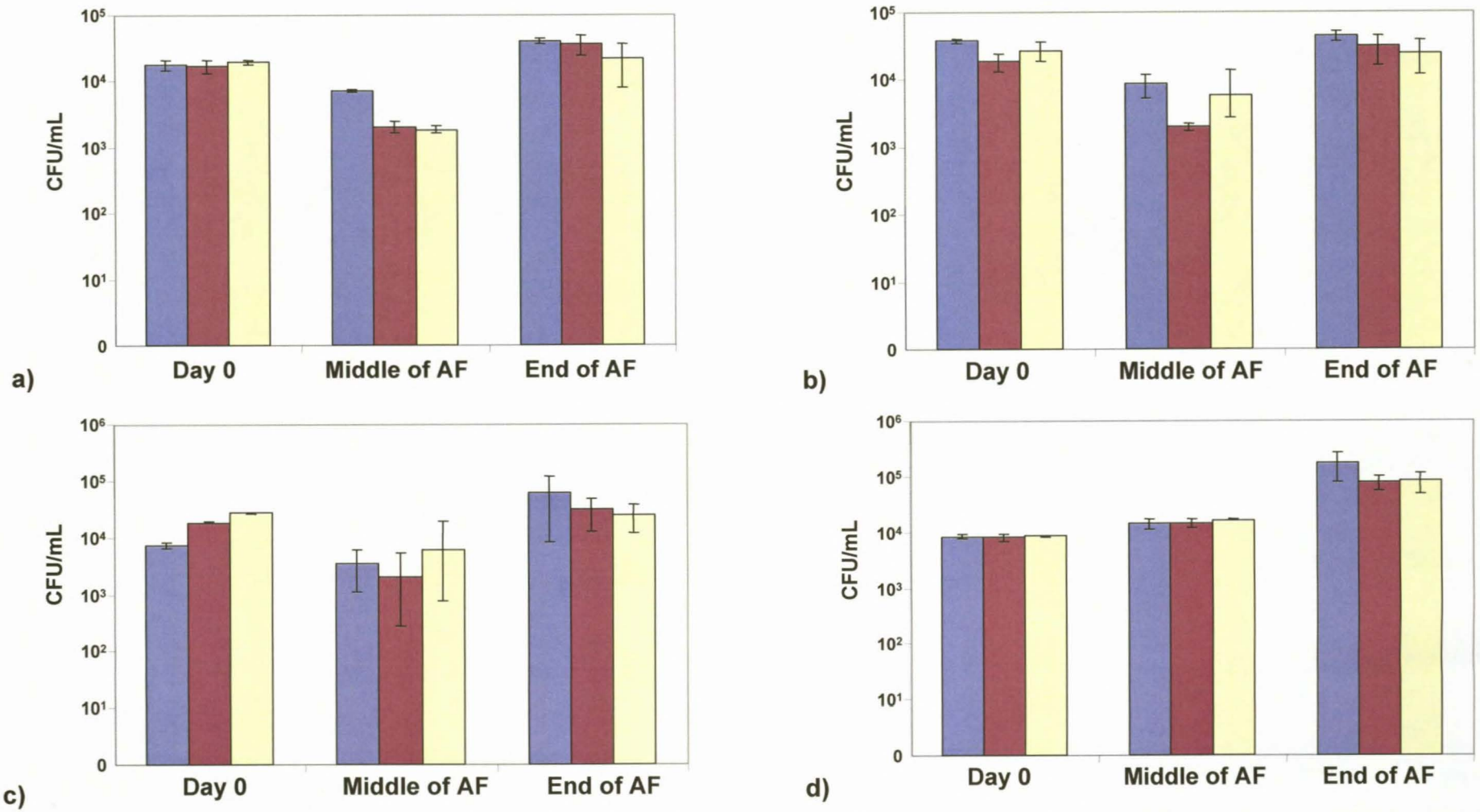


Figure 3.56. The effect of lysozyme on AAB (GYC and CARR media) and LAB (MRS and MRS + apple juice (MRSA)) numbers during small-scale Shiraz vinifications. a) MRS; b) MRSA; c) GYC and d) CARR. ■ Control; ■ 125 mg/L lysozyme; ■ 250 mg/L lysozyme.

3.3.2.1 Results of colour analyses and phenolic content of lysozyme treated and untreated red wines

The wine colour density at the middle of AF showed that little difference was evident between the treated samples (**Fig. 3.57**). The 250 mg/L lysozyme treated wines seemed to have a higher wine colour density than 125 mg/L lysozyme wines at the middle of AF during the Pinotage AF. Also no difference in wine colour density was found at the middle of AF during Cabernet Sauvignon AF. 250 mg/L lysozyme showed a slightly lower wine colour density during the Shiraz AF (middle of AF). No difference in wine colour density was observed between the control and 250 mg/L lysozyme of the Pinotage samples. Interestingly, 250 mg/L lysozyme resulted in a higher wine colour density than 125 mg/L lysozyme (Pinotage).

No difference was observed between the control, 125 mg/L lysozyme and 250 mg/L lysozyme at the middle of Cabernet Sauvignon's AF (**Fig. 3.57**). The 250 mg/L treated wines recorded a higher wine colour density at the end of AF than the control and 125 mg/L lysozyme (Cabernet Sauvignon). Two hundred and fifty mg/L recorded a lower wine colour density during the middle of the Shiraz AF. The control and 125 mg/L showed no significant difference during the middle of the Shiraz AF. 250 mg/L lysozyme also resulted in a slightly lower wine colour density at the end of the Shiraz AF.

The wine colour hue of Pinotage showed no difference between the treated samples during the middle of AF (**Fig. 3.58**). Also no difference was observed at the end of AF (Pinotage). No differences in wine colour hue at the middle and at the end of AF of Cabernet Sauvignon and Shiraz were found (**Fig. 3.58**).

The 125 mg/L lysozyme and 250 mg/L lysozyme treatments recorded no difference in terms of the total phenols during the middle of AF for Pinotage (**Fig. 3.59**). The control recorded a higher total phenol content at this stage. At the end of AF 250 mg/L lysozyme recorded higher total phenol content than the control and 125 mg/L lysozyme (Pinotage). No difference was found between the samples of Cabernet Sauvignon at the middle of AF (**Fig. 3.59**). 250 mg/L also proved to record the highest total phenol content at the end of AF. Lower total phenols were recorded for lysozyme treated wines during the Shiraz AF (**Fig. 3.59**). 250 mg/L lysozyme recorded the lowest total phenol content at the end of AF.

Overall the wine colour density of the Cabernet Sauvignon and Shiraz were higher than that of Pinotage. The fact that the Pinotage grapes originated from a young vineyard could be an explanation for this tendency or be due to cultivar differences. Cabernet Sauvignon showed a slightly lower wine colour hue if compared to that of the Pinotage and Shiraz. The total phenols also differed between the different cultivars. Although the total phenols were significantly lower at the middle of AF for Pinotage and Shiraz, higher values than those of the control were obtained for 250 mg/L lysozyme during the Pinotage and Cabernet Sauvignon AF.

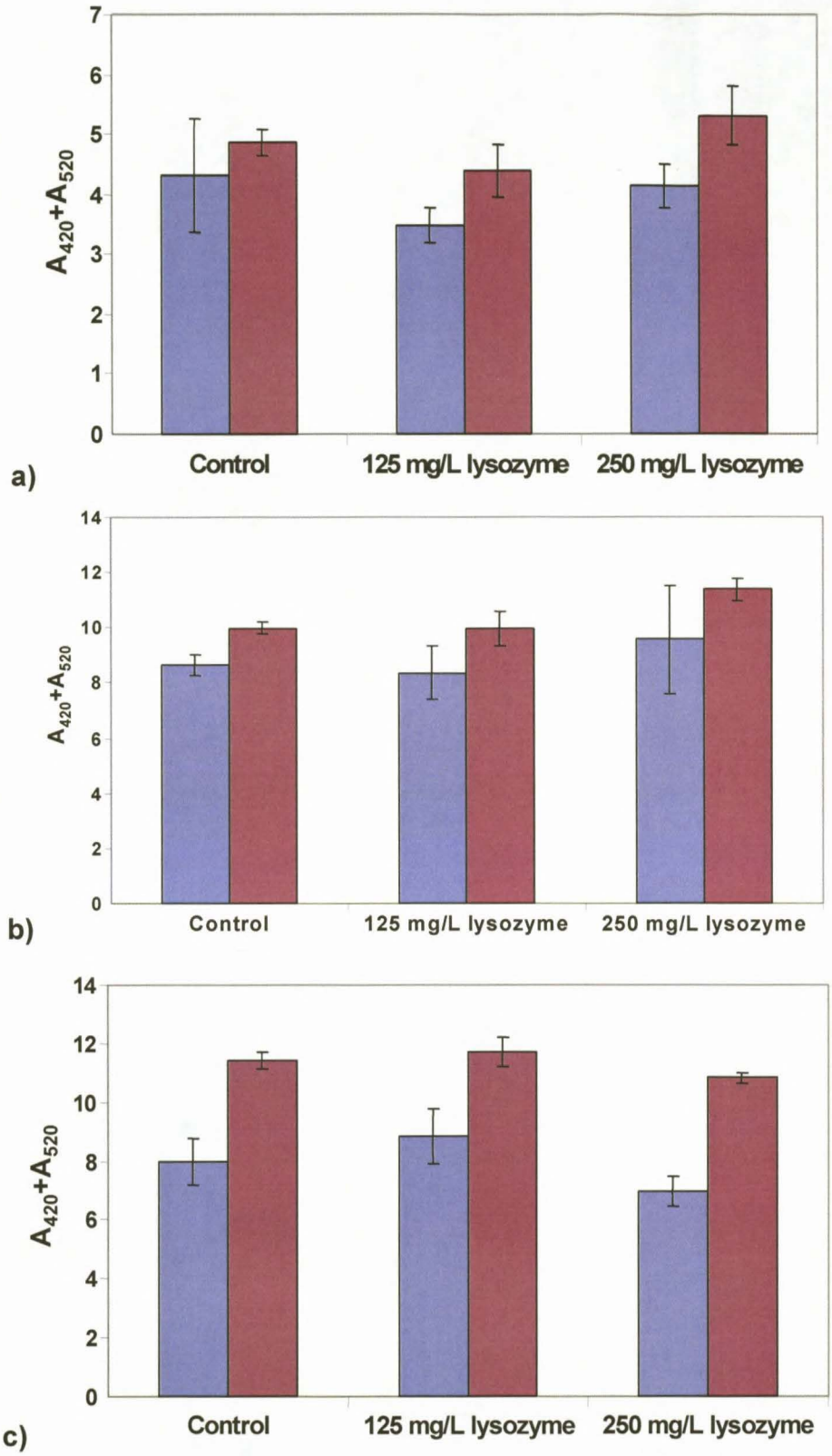
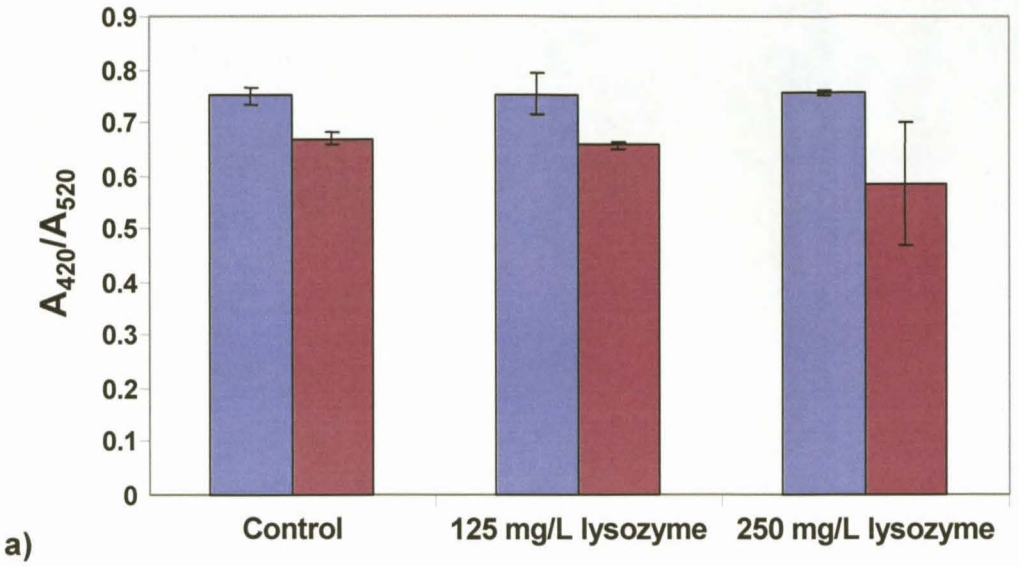
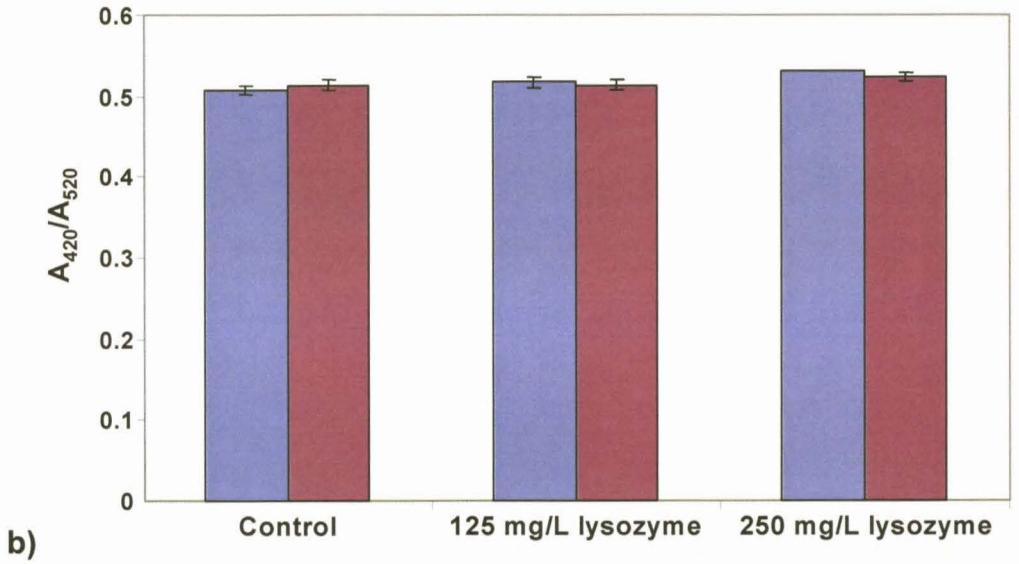


Figure 3.57. Wine colour density of a) Pinotage, b) Cabernet Sauvignon and c) Shiraz. ■ Middle of AF; ■ End of AF

No differences were also found with degree of red pigment colouration, estimate of SO_2 resistant pigments, total red pigments, modified wine colour density, modified wine colour hue, modified degree of red pigment colouration and modified estimate of SO_2 resistant pigments analysis (results not shown).



a)



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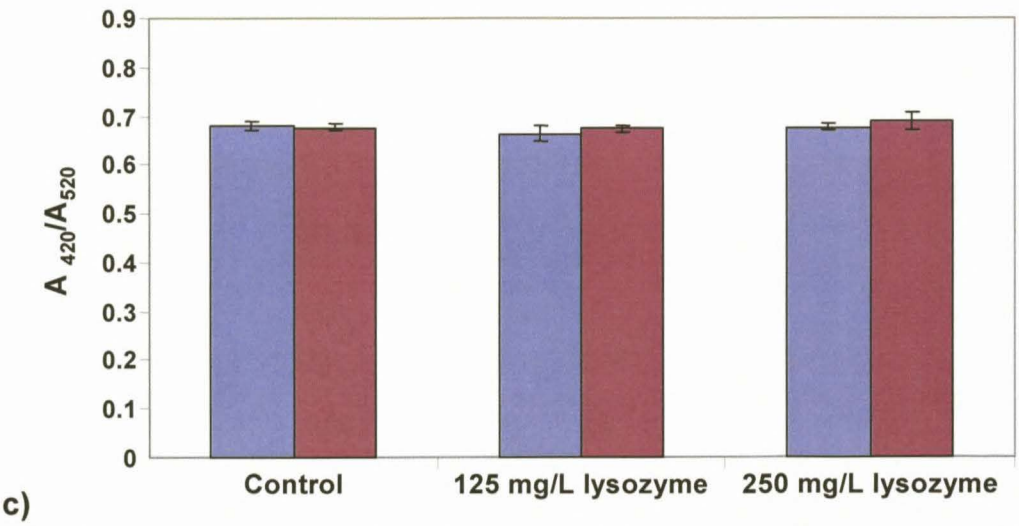

c)

Figure 3.58. Wine colour hue of a) Pinotage, b) Cabernet Sauvignon and c) Shiraz. ■ Middle of AF; ■ End of AF.

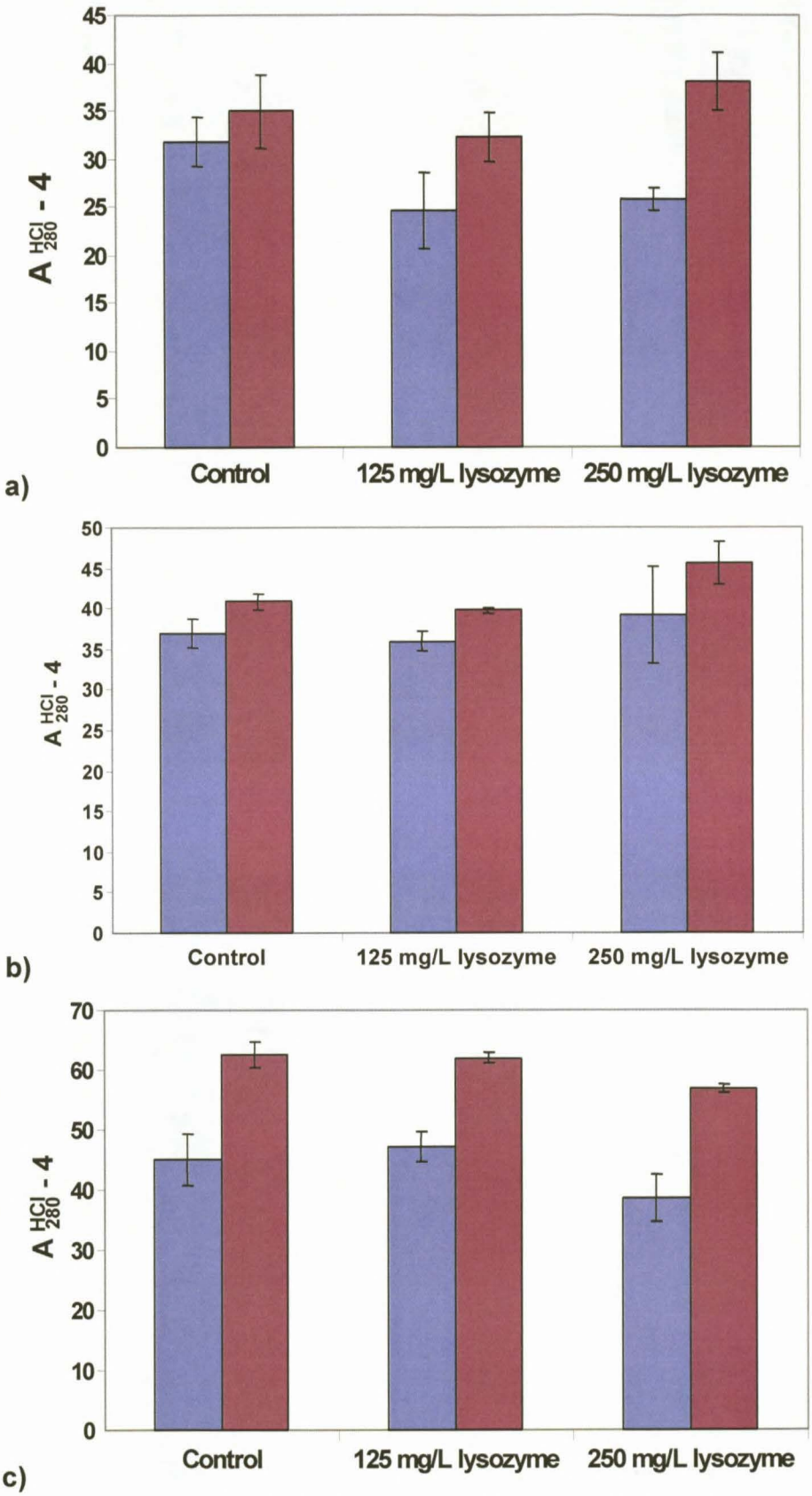

Figure 3.59. Total phenols (a.u.) of a) Pinotage, b) Cabernet Sauvignon and c) Shiraz. ■ Middle of AF; ■ End of AF.

3.3.2.2 The effect of lysozyme on the biogenic amine content of red wines

LAB are mainly responsible for the formation of biogenic amines in wines and musts by decarboxylation of the corresponding amino acids (Morena-Arribas *et al.*, 2003). It was thought that lysozyme addition would influence the occurrence of these substances during and after AF by inhibiting the growth of LAB. The three most frequently isolated biogenic amines in wine include histamine, tyramine and putrescine (Morena-Arribas *et al.*, 2003). *Pediococcus* spp. mainly produces histamine (Delfini, 1989), *O. oeni* can produce tyramine and histamine (Choudhury *et al.*, 1990; Lonvaud-Funel & Joyeux, 1994) and also *Lactobacillus brevis* strains were associated with tyramine formation in wine (Moreno-Arribas & Lonvaud-Funel, 1999; Morreno-Arribas *et al.*, 2000). Downing (2003) evaluated several LAB for biogenic amine production and found that most *L. hilgradii* strains produced biogenic amines as well as two strains of *L. brevis*.

The three tested cultivars did not show any significant differences between treatments (**Fig. 3.60**). In the case of the Pinotage and the Cabernet Sauvignon, slightly higher levels of putrescine were found at the end of AF for the two different lysozyme concentrations. However, a slight difference was observed between 125 mg/L lysozyme and 250 mg/L lysozyme in the case of Pinotage. The control showed slightly lower numbers of putrescine for Pinotage. These findings do not correspond to LAB numbers found during the AF of Pinotage (**Fig. 3.54**) where no difference between the control and lysozyme treated wines could be found. No difference of cadaverine levels was found between the control and lysozyme additions for all three different cultivars.

The occurrence of putrescine in Cabernet Sauvignon was found to be higher with 250 mg/L lysozyme addition while no difference was observed between the control and 125 mg/L lysozyme. Cabernet Sauvignon also recorded the highest putrescine levels of the three tested cultivars while Shiraz recorded the lowest values. This is probably due to the different precursors found at different levels/concentrations between the cultivars. When studying the LAB numbers found during the AF of Cabernet Sauvignon (**Fig. 3.55**) it can be argued that the levels of biogenic amines for both lysozyme treatments should be lower than that of the control. A possible explanation for this could be that the biogenic amines were formed during the first stages of AF in a rich amino acid environment.

No difference between the control and lysozyme additions was found in terms of cadaverine content for Pinotage, Cabernet Sauvignon and Shiraz. No difference was observed between the control and lysozyme additions during the Shiraz AF. No cadaverine was found for the three tested lysozyme concentrations in Shiraz, again showing that cultivar may play a role in biogenic amine content and type of biogenic amine found/produced or that that specific precursor is absent in that particular cultivar or grape variety.

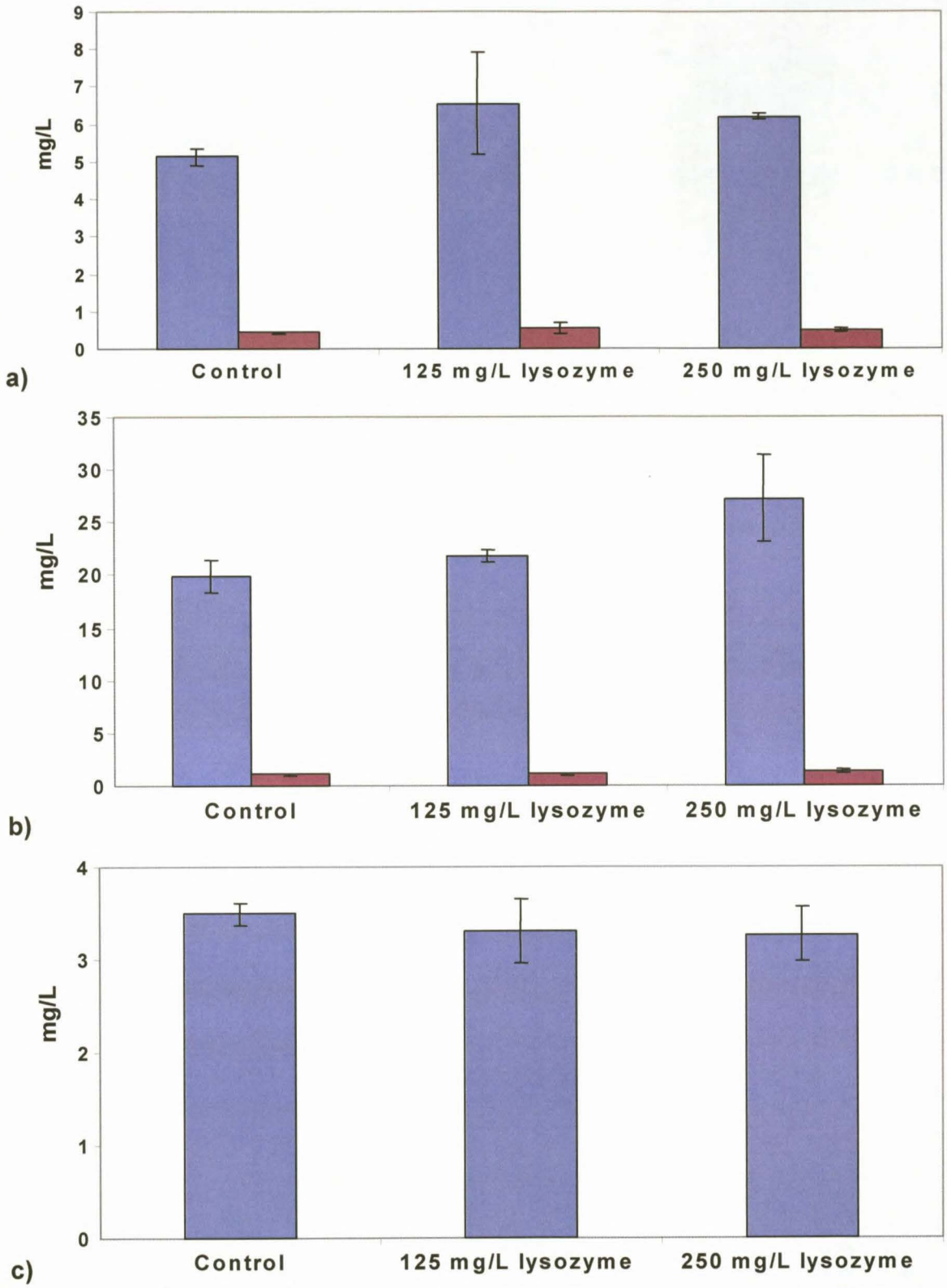

Figure 3.60. The effect of lysozyme on the biogenic amine content of a) Pinotage; b) Cabernet Sauvignon and c) Shiraz small-scale red wine vinifications measured at the end of AF. ■ putrescine; ■ cadaverine.

The effect of lysozyme addition on the biogenic amine production could not be evaluated completely in this experiment and needs further investigation. Moreover, this experiment would be best illustrated when these levels are investigated afterMLF, which is known for the phase where it increases the most.

3.3.2.3 RESULTS OF THE VOLATILE COMPONENTS

Unfortunately the Cabernet Sauvignon did not complete MLF after several weeks, and to exclude any potential oxidation or spoilage, only the Pinotage and Shiraz values were obtained, analysed and subsequently illustrated in this section. This tendency was observed in both the control as well as the lysozyme treated wines for Cabernet Sauvignon.

A major difference in acetoin content pre-MLF and post-MLF was observed in both Pinotage and Shiraz. In contrast to Pinotage, the Shiraz wines showed an increase in acetoin concentrations after MLF was completed. Bartowsky & Henscke (2004) reported that the formation of acetion and 2,3-butanediol from diacetyl is encouraged by the continued presence of bacteria or yeast lees following the completion of malic acid degradation (Cogan, 1987; Ramos *et al.*, 1995). Also, the timing of the sampling after MLF could have been different to that of the Shiraz samples.

The highest lysozyme concentration in Pinotage resulted in slightly lower acetoin level after AF and the same tendency can be seen with the post-MLF values. These differences were significant for the Pinotage wines. The particular grape variety could have influenced these findings. This can be seen in the post AF values between Pinotage and Shiraz where an average difference of four to five milligram per litre acetoin was found (**Fig. 3.61**). As mentioned before the Shiraz wines showed an increase in acetoin content after MLF. This was the case with the control

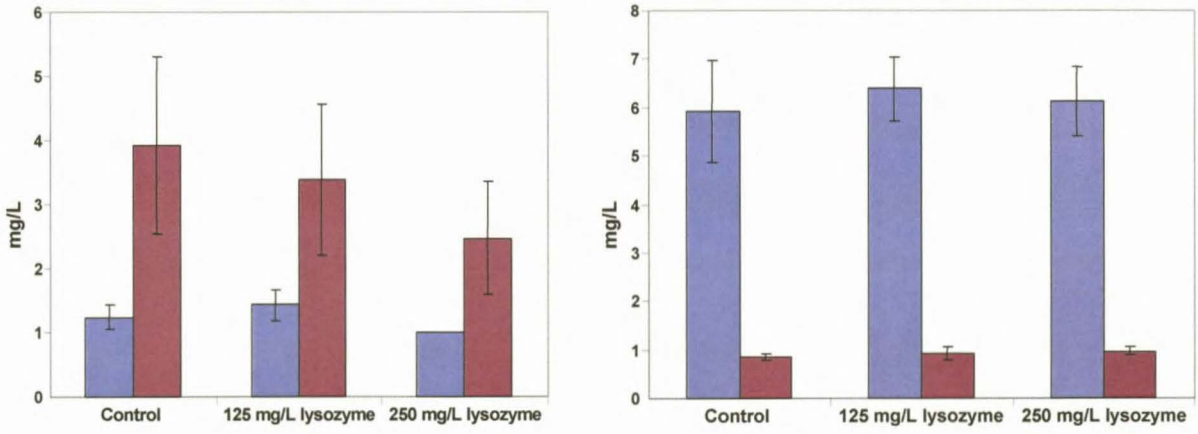

Figure 3.61. Acetoin concentrations in Pinotage (left) and Shiraz (right). ■; Post-MLF ■ End of AF.

and the two lysozyme concentrations, thus indicating that no difference between treatments is evident.

No difference in ethyl acetate concentration (**Fig. 3.62 appendix**) between the control and lysozyme treated wines could be seen pre-MLF and post-MLF for Pinotage and Shiraz. Du Plessis *et al.* (2004) found a decrease in ethyl acetate levels in brandy base wines that had undergone MLF. The values however varied immensely between cultivar with Pinotage recording between 60 to 80 mg/L in contrast to Shiraz's 15 to 25 mg/L.

No difference in ethyl hexanoate concentration (**Fig. 3.63 appendix**) and ethyl caprylate (figure not shown) was found for both Pinotage and Shiraz (figure not shown) between the control and lysozyme treated wines could be seen for both grape varieties. The ethyl hexanoate values of the Shiraz's post-MLF are much lower than that of the tested Pinotage wine.

An increase in ethyl lactate concentration after MLF was observed for both Shiraz and Pinotage wines (**Fig. 3.64**) corresponding to Du Plessis *et al.* (2004) who also found a significant increase in ethyl lactate concentrations of brandy base wines after MLF. Again the initial levels of ethyl lactate in the Pinotage wines were much higher than those of the Shiraz wines. No difference could be observed between the control and lysozyme treatments before and after MLF for both cultivars although 250 mg/L lysozyme seemed to record slightly lower values.

No differences in terms of hexanol could be observed between the control and lysozyme treated wines although concentrations seem to increase during MLF (**Fig. 3.65 appendix**). Hexyl acetate concentrations increased during MLF in Pinotage but remained unchanged in the Shiraz wines (**Fig. 3.66 appendix**). Overall higher levels of hexyl acetate were found in the Shiraz wines when compared to the Pinotage wines.

A definite decrease in the iso-amyl acetate concentration during MLF can be seen in **Figure 3.67**. Du Plessis *et al.* (2004) also found a decrease in iso-amyl acetate in brandy base wines that had undergone MLF. No difference between the

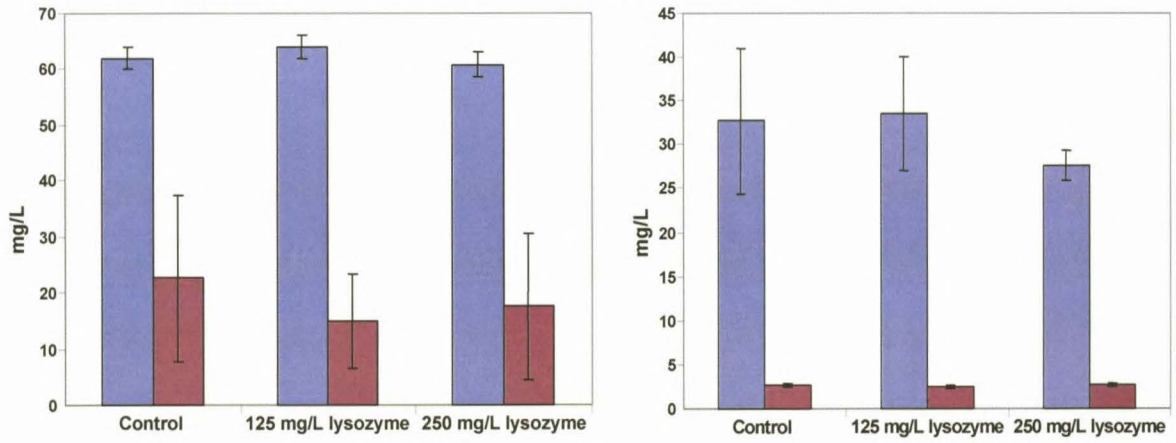

Figure 3.64. Ethyl lactate concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

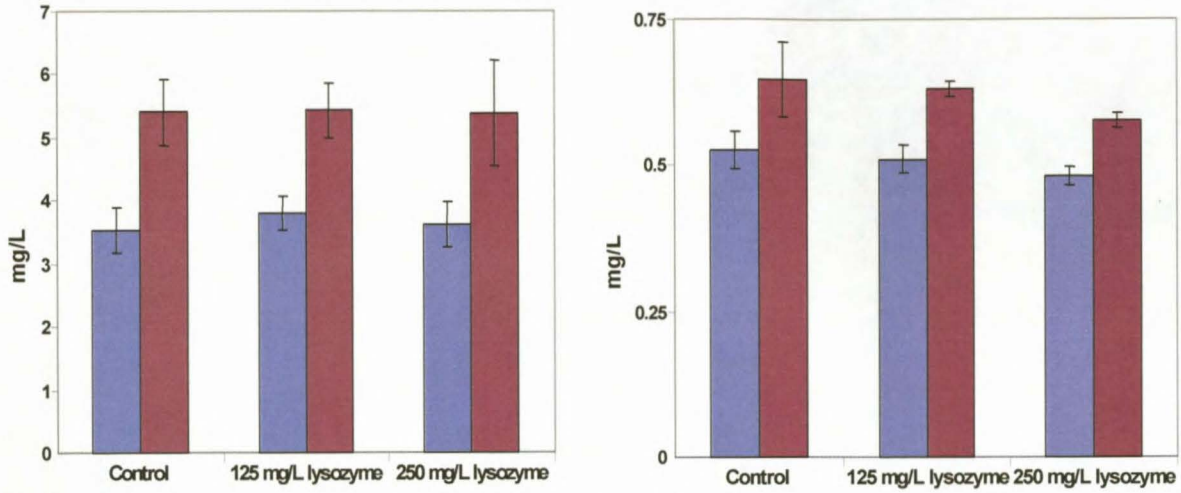

Figure 3.67. Iso-Amyl acetate concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

control and lysozyme treated wines was observed. A slightly lower post-MLF value was recorded with 250 mg/L lysozyme in the Shiraz samples.

No difference in the iso-amyl alcohol, iso-butanol and methanol content between the control and lysozyme treated wines was observed, except for 250 mg/L lysozyme that showed a slightly lower methanol value after MLF in the Pinotage sample (**Fig. 3.68; 69; 70 appendix**).

n-Butanol showed a slightly lower value after MLF in the Pinotage wine, but there was no difference between the control and lysozyme treated wines. Shiraz showed almost no difference in pre-MLF and post-MLF values (**Fig. 3.71 appendix**). n-Propanol was slightly higher with the 250 mg/L lysozyme addition than the control and 125 mg/L addition, especially post MLF. No difference was found between the treatments in the Shiraz wines. All the samples showed an increase in acetic acid after MLF for both cultivars. Pinotage recorded the highest acetic acid content (440 mg/L for the control) after MLF in contrast to Shiraz (135 mg/L for the control). No difference was observed between the control and lysozyme treated wines. A slight increase in diethyl succinate was found in wines that had undergone MLF (results not shown). Du Plessis *et al.* (2004) also found an increase in acetic acid and diethyl succinate during MLF in brandy base wines.

3.4 DISCUSSION

The objective of the small-scale Chenin blanc fermentation show that lysozyme is effective in inhibiting or lowering LAB cell numbers during a controlled AF. However, several strains illustrated partial or complete resistance to lysozyme. This was evident for only *Lactobacillus* strains such as *L. buchneri* V1 , *L. brevis* J23; 81.1 and *L. paracasei* L43. Our results are contradictory to those of Gao *et al.* (2002) who found no resistant LAB to lysozyme treatments, however only four strains were evaluated in their study. Interestingly, the type strain of *L. buchneri* illustrated complete sensitivity to the tested lysozyme concentrations. Several factors could be argued to have had an influence on these results. This could indicate that the native/locally isolated strains of LAB are more adapted to local wine conditions than some of the type strains that was not isolated from an wine environment. Also, only wine isolates showed partial or complete resistance to lysozyme treatments. It would also be interesting to evaluate these more resistant LAB strains in the presence of a higher molecular free fraction of SO_2, and by doing this, complement the action of lysozyme. The fact that only four strains out of the total 26 tested showed resistance to lysozyme is encouraging.

On the other hand, the majority of the tested strains illustrated partial or complete sensitivity to lysozyme. Complete sensitivity to lysozyme was seen during the alcoholic fermentation of *L. nagelii* ATCC70062^T, *L. pentosus* DSM 20314^T, *L. vermiforme* W16; NCDO 962, *L. paraplantarum* # 101, *L. hilgardii* M52, *L. plantarum* K57; # 50, *L. paracasei* # 54; DSM 5622^T and *L. buchneri* DSM 20057^T. Of these, only *L. buchneri* DSM 20057^T and *L. paracasei* DSM 5622^T exhibited no net growth during the AF. No re-growth of LAB after lysozyme inhibition was observed during the AF except for *L. hilgardii* M52.

The strains that did not show positive growth during the alcoholic fermentation includes *L. plantarum* LMG 13556, *L. fermentum* LMG 13554, *P. acidilactici* PAC 1.0; # 118, *L. pentosus* # 42 and *Leuconsotoc mesenteroides* # 5. However a decrease in cell numbers could be observed for both the control and lysozyme treated samples, it would be difficult to prove that lysozyme was solely responsible for the reduction in numbers of especially *L. plantarum* LMG 13556, *L. fermentum* LMG 13554 and *Leuconsotoc mesenteroides* # 5. Also, the fact that the first enumerations were done after day three of AF could have indicated different results. It can be argued that other factors in the must could have influenced the growth of the tested LAB. Factors such as alcohol production during AF, SO_2 concentration (however slight), pesticides (not tested), fatty acids produced by yeast, some traces of residual dimethyl dicarbonate (although the bulk of the juice was divided among the individual fermentation flasks) and insufficient nutrients could have had a negative effect on cell growth during AF. The possibility that phenols could have reacted with lysozyme is slight due to the fact that white grape juice was used, which contains low amounts of phenolics.

Strains that showed an initial increase followed by a decrease in cell numbers but failed to be inhibited during the AF by lysozyme included *L. paracasei* #84, *L. plantarum* #14. *L. pentosus* K22 and *L. sakei* LMG 13558 showed a reduction in numbers during the first three days of AF when treated with lysozyme, but continued to survive during the AF. It would have been interesting to evaluate these strains over an extended period of time.

It is clear that lysozyme did not have any negative affect on the AFT. However, the AFT of *L. sakei* LMG 13558 and *L. pentosus* DSM 20314^T illustrated contradictory results. An improved AFT was observed for the control when compared to the lysozyme treated samples. No difference was observed between the control and 250 mg/L lysozyme with 500 mg/L lysozyme resulting in a slower AFT. However, these were the only AF to show these results. A slight improvement in AFT was observed in the case of *L. nagelii* ATCC70062^T, *L. vermiforme* NCDO 962, *L. paracasei* #84, *L. pentosus* #42 and *L. paracasei* #54 when lysozyme was added.

The effect of lysozyme on LAB and AAB numbers during red wine vinifications showed contradictory results between the different cultivars. The effect of lysozyme on LAB numbers during the Pinotage and Shiraz AF was not significant. During the Pinotage AF the decrease in LAB numbers could not be attributed to lysozyme due to the control that also illustrated a reduction in LAB numbers over time. On both LAB enumeration media no differences could be observed. The natural flora of LAB could have been sensitive to the effect of ethanol, however, other factors could also play a role such as SO_2 (addition and/or production by yeast), pH (although the initial values were pH > 4 and should favour the growth of LAB, especially *Lactobacillus* and *Pediococcus* species), phenolic compounds, fatty acids, temperature, pesticides etc.

It was proven that the activity of lysozyme increases with an increase in pH (eg. pH > 4) thus it would, from a scientific perspective, favour the action of lysozyme during the Shiraz AF. However, contradictory results were obtained with no reduction in numbers during the AF. No difference could be seen between the control and lysozyme treated wines. Cultivar and phenolic composition of Shiraz could have influenced the result. The LAB numbers of the Shiraz fermentation differs to that of the Pinotage and Cabernet Sauvignon fermentations. Hence, the specific cultivar could influence to action of lysozyme. Other researchers also found that the specific cultivar (eg. different phenolic composition) could influence the action of lysozyme (Lagarde, personal communication 2003).

Lysozyme addition during the Cabernet Sauvignon AF illustrated excellent results with a reduction of LAB numbers at the end of AF. Significant differences were seen between the control and lysozyme treated wines. However, when Cabernet Sauvignon was inoculated with a starter culture to conduct MLF, no MLF could be initiated. The reasons could be residual lysozyme concentrations in the wine, which is, however, unlikely due to the fact that the control also did not complete MLF.

The second part of the study indicates that lysozyme does not cause an increase in AAB numbers during the AF. Two lower lysozyme concentrations were chosen to

represent the dosages commonly used in winemaking. No difference could be seen in all three tested cultivars on two different media while contradictory results were found for LAB numbers. Various factors could influence the natural AAB flora of the grapes (Du Toit & Pretorius, 2002). Du Toit & Lambrechts (2002) reported that high numbers of AAB can be correlated with the initial pH of the must and that cold soaking favours the growth of AAB at higher pH levels which in this case was evident. Moreover, only a small amount of SO_2 was used at crushing. This could be an explanation for the high AAB numbers. The genera of natural LAB and AAB species that are present on the grapes could also have differed between the tested cultivars. This study showed that lysozyme addition does not have any effect on the Gram-negative populations found in musts and wine even if the Gram-positive organisms are inhibited.

Colour and phenolic content differed between the tested cultivars. It is clear that lysozyme did not result in a decreased colour and phenol content when added at the start of AF. None of these three cultivars are known to produce lightly coloured wines as is the case with the cultivar Pinot noir. Lightly coloured and elegant wines (for instance Pinot noir) could be influenced by the addition of lysozyme. Hence, researchers have studied the influence of lysozyme on especially Pinot noir in the Rhône valley. They found that when lysozyme is added during certain vinification stages, that no effect on colour is evident (Lagarde, personal communication 2003). Overall South Africa is not renowned for Pinot noir, however, it would have been interesting to evaluate the effect of lysozyme on South African grown and made Pinot noir at different stages of vinification.

The effect of lysozyme on the biogenic amine content could not be observed and needs further investigation. It would have been interesting to analyse the laboratory wines for differences in biogenic amine content. However, differences were found in the amount of biogenic amine between cultivar, thus indicating that the amount produced is cultivar dependant. It is possible that the specific amino acid precursor differs between cultivars. Only putrescine and cadaverine were found in all three tested cultivars.

The volatile components showed that no major differences were observed between the different treatments for Shiraz and Pinotage during the AF and after MLF. This correlates back to the fact that no major differences in LAB and AAB numbers were found between treatments during the AF for those tested cultivars. Acetoin concentrations seemed to decrease during MLF in Pinotage but increased during MLF in the tested Shiraz wines. Ethyl lactate increased during MLF or all the treatments but no differences could be seen between treatments. All the treatments showed a decrease in iso-amyl acetate during MLF. It would have been interesting to evaluate the results obtained from Cabernet Sauvignon to correlate any difference in volatile components back to the difference in CFU/mL numbers during the AF and possibly MLF.

3.5 CONCLUSIONS

This study showed that mainly wine isolated *Lactobacillus* strains are more resistant to lysozyme concentrations during a controlled small-scale AF. Also, some of the tested LAB strains illustrated partial resistance to lysozyme. However, the majority of LAB species illustrated complete inhibition to the action of lysozyme. Lysozyme had no effect on the AFT and did not result in an increase in AAB numbers if added at the start of AF. No effect on the colour and phenol content was observed on three tested red cultivars when lysozyme was added at the start of the AF. No conclusion regarding the influence of lysozyme on biogenic amine content and volatile components could be drawn from this study and needs further investigation.

3.6 ACKNOWLEDGEMENTS

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3.7 LITERATURE CITED

Alberto, M.R., Arena, M.E. Manca de Nadra, M.C., 2002. A comparative survey of two analytical methods for identification and quantification of biogenic amines. Food Control 13, 125-129.

Arima, H., Ibrahim, H.R., Kinoshita, T. & Kato, A., 1997. Bacterial action of lysozyme attached with various sizes of hydrophobic peptides to the C-terminal using genetic modification. Fed. Europ. Biochem. Soc. 415, 114-118.

Bartowsky, E.J. & Henschke, P.A., 2004. The 'buttery' attribute of wine-diacetyl-desirability, spoilage and beyond – Review article. Int. J. Food Microbiol. 96, 235-252.

Carreté, R., Vidal, M.T., Bordons, A. & Constantí, M., 2002. Inhibitory effect of sulphur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. FEMS Microbiol. Lett. 211, 155-159.

Choudbury, N., Hansen, W., Engesser, D., Hammes, W.P. & Holzapfel, W.H., 1990. Formation of histamine and tyramine by lactic acid bacteria in decarboxylase assay medium. Lett. Appl. Microbiol. 11, 278-281.

Cogan, T.M., 1987. Co-metabolism of citrate and glucose by *Leuconostoc* spp.: effects of growth, substrates and products. J. Appl. Bacteriol. 63, 551-558.

De Man, J.C., Rogosa, M. & Sharpe, M.E., 1960. Medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23, 130-138.

Delfini, C., 1989. Ability of wine malolactic bacteria to produce histamine. Sci. Aliments 9, 413-416.

Delfini, C., Gaia, P., Schellino, R., Strano, M., Pagliara, A. & Ambrò, S., 2002. Fermentability of grape must after inhibition with dimethyl dicarbonate (DMDC). J. Agric. Food Chem. 50, 5605-5611.

Dicks, L.M.T., Dellaglio, F. & Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig] gen.nov., comb. nov. Int. J. Syst. Bacteriol. 45, 395-397.

Drysdale, G.S. & Fleet, G.H., 1988. Acetic acid bacteria in winemaking: A Review. Am. J. Enol. Vitic. 39, 143-154.

Downing, L., 2003. Characterisation of biogenic amine genes in lactic acid bacteria isolated from wine. Masters thesis, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa.

Du Plessis, H.W., Dicks, L.M.T., Pretorius, I.S., Lambrechts, M.G. & Du Toit, M., 2004. Identification of lactic acid bacteria isolated from South African brandy base wines. Int. J. Food Microbiol. 91, 19-29.

Du Toit, W.J. & Lambrechts, M.G., 2002. The enumeration and identification of acetic acid bacteria from South African red wine fermentations. Int. J. Food Microbiol. 74, 57-64.

Du Toit, M. & Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - A Review. S. Afr. J. Enol. Vitic. 21, Special Issue, 74-96.

Edwards, C.G. & Jensen, K.A., 1992. Occurrence and characterization of lactic acid bacteria from Washington state wines: *Pediococcus* spp. Am. J. Enol. Vitic. 43, 233-238.

Edwards, C.G., Collins, M.D., Lawson, P.A. & Rodriguez, A.V., 2000. *Lactobacillus nagelii* sp. nov., an organism isolated from partially fermented wine. Int. J. Syst. Evol. Microbiol. 50, 699-702.

Fleet, G.H., 1998. The Microbiology of Alcoholic Beverages. Microbiology of Fermented Foods Vol. 1, Blackie Academic & Professional, London.

Gao, Y-C., Zhang, G., Krentz, S., Darius, S., Power, J. & Lagarde, G., 2002. Inhibition of spoilage lactic acid bacteria by lysozyme during wine alcoholic fermentation. Aus. J. Grape Wine Res. 8, 76-83.

Gerbaux, V., Villa, A., Monamy, C. & Bertrand, A., 1997. Use of lysozyme to inhibit malolactic fermentation and to stabilize wine after malolactic fermentation. Am. J. Enol. Vitic. 48, 49-54.

Guerrini, S., Mangani, S., Granchi, L. & Vincenzini, M., 2001. Biogenic amine production by *Oenococcus oeni*. Curr. Microbiol. 44, 374-378.

Henick-Kling, T., Acree, T. & Edinger, W., 1998. Sensory impact of malolactic fermentation. Intervitis, 11-17 May, Stuttgart, Germany, 193-197.

Huang, Y.C., Edwards, C.G., Peterson, J.C. & Haag, K.M., 1996. Relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. Am. J. Enol. Vitic. 47, 1-10.

Iland, P., Ewart, E.A. & Sitters, J., 1993. Techniques for chemical analysis and stability tests of grape juice and wine. Patrick Iland Wine Promotions, Adelaide, Australia.

Iland, P., Ewart, E., Sitters, J., Markides, A. & Bruer, N., 2000. Techniques for chemical analysis and quality monitoring during winemaking. Patrick Iland Wine Promotions, Campbelltown, South Australia, Australia.

JianFen, L. & Yong, L., 2004. Effect of natamycin on growth of yeast. J. China Agric. Uni. 9, 96-99.

Joyeux, A., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984a. Evolution of acetic acid bacteria during fermentation and storage of wine. Appl. Environ. Microbiol. 48, 153-156.

Joyeux, A., Lafon-Lafourcade, S. & Ribéreau-Gayon, P., 1984b. Metabolism of acetic acid bacteria in grape must: consequences on alcoholic and malolactic fermentation. Sci. Aliments 4, 247-255.

Kantz, K. & Singleton, V.L., 1991. Isolation and determination of polymeric polyphenols in wines using sephadex LH-20. Am. J. Enol. Vitic. 42, 309-316.

Krieling, S.J., 2003. An investigation into lactic acid bacteria as possible cause of bitterness in wine. Masters thesis, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa.

Lilly, M., Lambrechts, M.G. & Pretorius, I.S., 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. Appl. Environ. Microbiol. 66, 744-753.

Lafon-Lafourcade, S., Carre, E. & Ribéreau-Gayon, P., 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl. Environ. Microbiol. 46, 874-880.

Lonvaud-Funel, A. & Joyeux, A., 1994. Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc oenos*. J. Appl. Bacteriol. 77, 401-407.

Lonvaud-Funel, A., Joyeux, A. & Desens, C., 1988. Inhibition of malolactic fermentation of wines by products of yeast metabolism. J. Sci. Food Agric. 44, 185-191.

Lonvaud-Funel, A., 1995. Microbiology of the malolactic fermentation: molecular aspects. FEMS Microbiol. Lett. 126, 209-214.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek, 76, 317-331.

Maicas, S., Gil, J., Pardo, I. & Ferrer, S., 1999. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food Res. Int. 32, 491-496.

McKenzie, H.A. & White, F.H., 1991. Lysozyme and α-lactalbumin: Structure, function, and interrelationships. Adv. Prot. Chem. 41, 173-315.

Moreno-Arribas, M.V. & Lonvaud-Funel, A., 1999. Tyrosine decarboxylase activity of *Lactobacillus brevis* IOEB 9809 isolated from wine and *L. brevis* ATCC 367. FEMS Microbiol. Lett. 195, 103-107.

Moreno-Arribas, M.V., Polo, M.C., Jorganes, F. & MuÑoz, R., 2003. Screening of biogenic amine production by lactic acid bacteria from grape must and wine. Int. J. Food Microbiol. 84, 117-123.

Moreno-Arribas, M.V., Torlois, S., Joyeux, A., Bertrand, A. & Lonvaud-Funel, A., 2000. Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine. J. Appl. Microbiol. 88, 584-593.

Pilatte, E., Naigaard, M., Gao, Y., Krentz, S., Power, J. & Lagarde, G., 2000. Study of the effect of lysozyme on different strains of *Oenococcus oeni*. Applications in the management of malolactic fermentation. Originally published in French in Revenue Française d' Oenologie.

Ramos, A., Lolkema, J.S., Konings, W.N. & Santos, H., 1995. Enzyme basis for the pH regulation of citrate and pyruvate metabolism by *Leuconostoc oenos*. Appl. Environ. Microbiol. 61, 1303-1310.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000a. The Microbiology of Wine and Vinifications. Handbook of Enology, vol. 1, John Wiley & Sons Ltd.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000b. The Chemistry of Wine and stabilization and Treatments. Handbook of Enology, vol. 2, John Wiley & Sons Ltd.

Ruiz, A., Poblet, M., Mas A. & Guillamon, J.M., 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. Int. J. Syst. Microbiol. 50, 1981-1987.

Splittstoesser, D.F. & Churney, J.J., 1992. The incidence of sorbic acid-resistant *Gluconobacters* and yeasts on grapes grown in New York State. Am. J. Enol. Vitic. 43, 290-293.

Stiles, M.E. & Holzapfel, W.H., 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1-29.

Stratiotis, A.L. & Dicks, L.M.T., 2002. Identification of *Lactobacillus* spp. isolated from different phases during the production of a South-African fortified wine. S. Afr. J. Enol. Vitic. 23, 14-21.

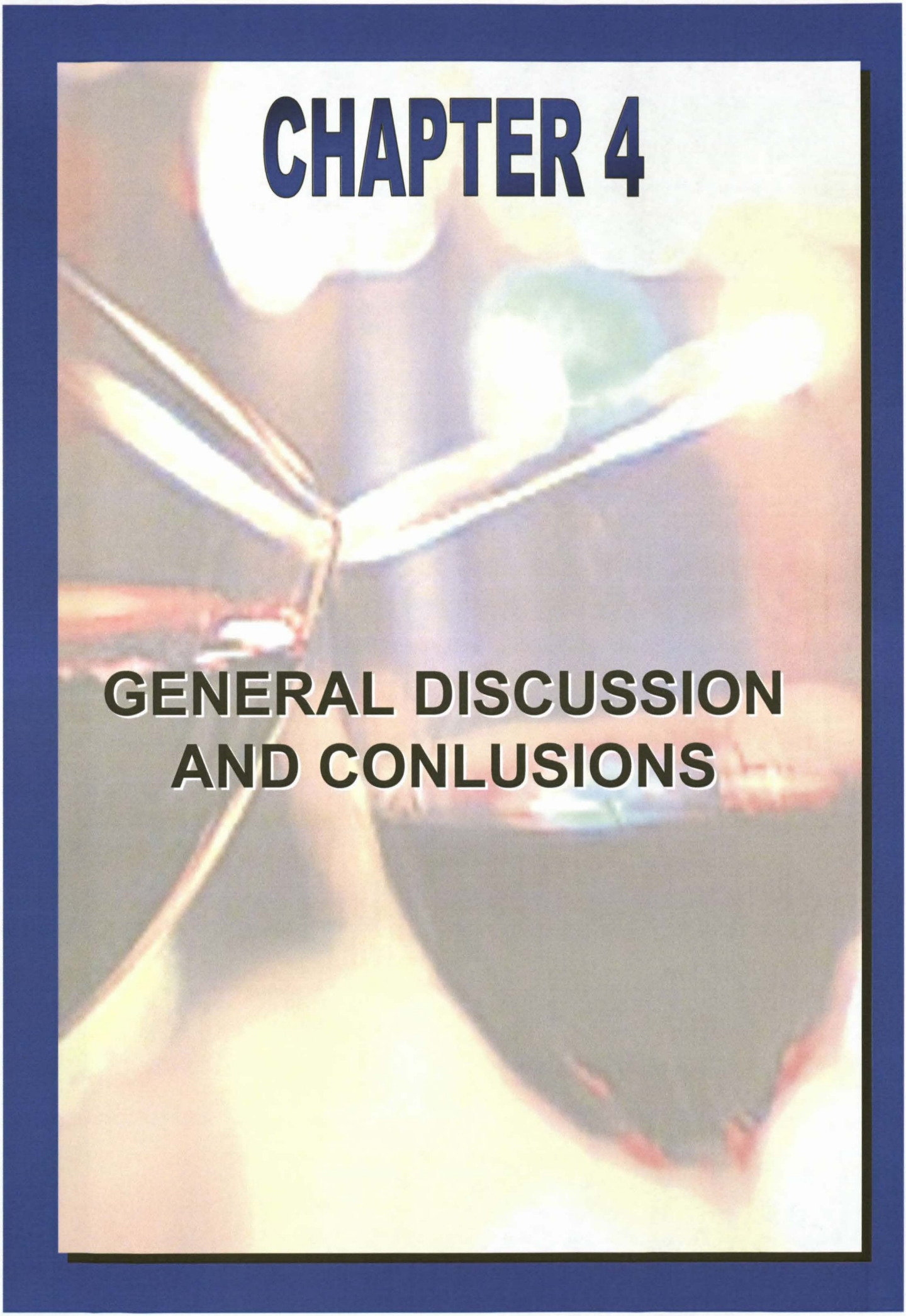
Tchelistcheff, A., Peterson, R.G. & Van Gelderen, M., 1971. Control of malo-lactic fermentation in wine. Am. J. Enol. Vitic. 69 1-5.

Wilkinson, B.R. & Dorrington, R.E., 1975. Lysozyme (murimidase) from waste egg white. Process Biochem. 10, 24.

Yamada, Y., Hoshino, K. & Ishikawa, T., 1997. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: The elevation of the subgenus *Gluconobacter* to the generic level. Biosci. Biotech. Biochem. 61, 1244-1251.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nurry, F.S., 1995. Wine Analysis and Production. Chapman & Hall, New York.

Foss analytical http: www//foss.dk



CHAPTER 4

GENERAL DISCUSSION AND CONLUSIONS

4. GENERAL DISCUSSION AND CONCLUSION

4.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

A lot of emphasis in the modern era of winemaking is placed on quality and visual appearance of a wine. Competition is fierce between wine-producing countries and with an annual global overproduction of wine any instability or spoilage in/of wine could result in a bad image of a product. This can have a negative influence on any wine-producer or wine-producing country. Lactic acid bacteria (LAB), though associated with and responsible for MLF, are often regarded as spoilage microorganisms causing ropiness, volatile acidity (VA), biogenic amines, ethyl carbamate, bitterness, mannitol, geranium tone and sluggish and/or stuck alcoholic fermentation (AF) (Du Toit & Pretorius, 2000).

Species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus* are associated with the winemaking process (Lonvaud-Funel, 1999; Du Toit & Pretorius, 2000). During MLF, L(+)-malic acid is converted to L(+)-lactic acid and CO_2 (Kunkee, 1991; Lonvaud-Funel, 1999). The positive contribution on wine flavour by LAB (*O. oeni*) is the production of diacetyl, increased complexity and increased mouthfeel of wine. These positive effects normally occur after the completion of AF. Several authors differed in their opinion of the positive contributions of MLF to wine (Davis *et al.*, 1985; Bartowsky & Henscke, 2004). It has been shown that the occurrence of specific LAB species and/or LAB strains differs during the different stages of winemaking (Lafon-Lafourcade *et al.*, 1983; Stratiotis & Dicks, 2002). This could thus influence the final flavour profile of the wine, whether negatively or positively.

The commonly known factors that influence the growth and survival of LAB in wine include pH (Davis *et al.* 1986, 1988; Wibowo *et al.* 1988; Britz & Tracey, 1990), ethanol concentration (Carreté *et al.*, 2002; Ribéreau-Gayon *et al.*, 2000), temperature (Beelman *et al.*, 1977; Britz & Tracey, 1990; Ribéreau-Gayon *et al.*, 2000), SO_2 concentration (Davis *et al.*, 1986; 1988; Edwards *et al.*, 1999), phenolic compounds (Vivas *et al.*, 1997 ; 2000; Campos *et al.*, 2003), pesticides (Vidal *et al.*, 2001) and fatty acids (Lafon-Lafourcade *et al.*, 1984; King & Beelman, 1986).

Lysozyme, an enzyme extracted from hen egg white that it used to inhibit the growth of LAB, has been approved by the OIV for usage in wine and wine related products. However, several questions regarding the influence on LAB species and wine quality still need to be addressed and researched. One of these questions include if lysozyme is used during the AF, what is the potential of residual lysozyme, especially during white winemaking? This factor could also influence the naturally occurring LAB species and/or the growth and survival of inoculated LAB species. There have not been numerous studies on lysozyme in oenology with only a few authors publishing wine-related lysozyme results (Gao *et al.*, 2000). This could again be due to the fact that lysozyme was only approved by the OIV for usage in winemaking since 2000. With increased general sales of lysozyme all

over the world it is currently used in the European Union, North and South America and countries of the Southern Hemisphere.

In Chapter 1, the importance of this study was highlighted. The main aims of this study were to determine resistance levels of selected LAB species and strains to lysozyme, how it influenced the alcoholic fermentation tempo, the influence on acetic acid bacteria and lactic acid bacteria populations as well as on the physical parameters (colour, phenol content, biogenic amine content and volatile components).

Chapter 2 focuses on the microorganisms found during the winemaking process. Several factors that influence the growth and survival of LAB were discussed. Chemical preservation, physical stabilisation and sterilisation and biopreservation of juice and wine completed the third part of the literature review. The use of lysozyme in wine, as part of the biopreservation of wine, was the main focus of this study.

Chapter 3 describes and discusses the above mentioned issues. This study revealed that lysozyme is effective in lowering or inhibiting LAB numbers during a controlled AF. It was shown that only four out of twenty six evaluated LAB strains were resistant to the tested lysozyme concentrations. Partial or complete inhibition was observed for the remaining LAB species. In some of the alcoholic fermentations it would have possibly been better to evaluate the fermentations over an extended period (up to 30 days) of time.

Also, if possible, the first enumeration of LAB should be done during the first three days of AF, as seen with some of the LAB species which showed a zero CFU/mL count at day three. The assumption would be that these LAB were inhibited by lysozyme in less than three days of AF, however it would be, in this case, difficult to prove. Previous studies showed that some LAB species dominate at different (later) stages of AF.

Future studies could include the effect of oak/barrel on LAB numbers in combination with lysozyme, following the traditional barrel fermentation used for Chardonnay as well as MLF for red wines in barrel. However, too many parameters such as barrel differences could influence the result. It would then be better to test oak chips/staves in combination with lysozyme to mimic the effect of wood, but this would be a study on its own. Another difficulty would be to sterilise the oak chips/staves that would be used in such an experiment. Further experiments could also include commercial tannin additions and the effect it has on lysozyme due to the fact that winemakers add these tannins during different stages of winemaking. The quantification of the different phenols in these commercial tannin preparations could pose a problem. The reactions with different phenolic compounds could be a very difficult task due to the fact that red wine is very complex and that most of the tannins are in a polymerised form. This should be an intensive study on its own and cannot be illustrated with one or two experiments by using monomeric phenols such as catechin and epicatechin.

Another aspect, in terms of spoilage by LAB, is to analyse the fermented wines (laboratory scale fermentations) for volatile acidity and biogenic amine production to correlate the inhibited LAB's levels with the more resistant LAB species.

Larger volumes of fermented Chenin blanc could also have been used to accommodate comparative tastings between the different LAB species and lysozyme treatments, however then only a few LAB species should then be evaluated to accommodate the workload. Several other LAB species, such as *O. oeni*, were evaluated but no successful growth was achieved in the tested grape juice, thus this study cannot supply information about the sensitivity of *O. oeni* to lysozyme under South African conditions. Another study could include different company's starter cultures of *O. oeni* that is commonly used to conduct MLF in wines. No difference between the control and lysozyme treated wines was observed for the alcoholic fermentation tempo.

This study also showed that lysozyme does not lead to an increase in AAB numbers in red wine, although contrasting results were found in terms of the LAB numbers. It is difficult to achieve good results with non-sterile juice such as red juice/must and these results should be interpreted as tendencies only. However, red must/juice contains such a diversity of micro-organisms that it would also be difficult to simulate actual wine conditions in artificial juice with all the relevant microorganisms. Thus, the ideal for the AAB and LAB numbers experiments will better be illustrated over an extended period investigation, for example 5 years. No species identification was done on the isolated AAB and LAB. LAB numbers varied between the tested cultivars. Only one out of three cultivars showed positive results in terms of LAB inhibition by lysozyme during the AF.

More studies are needed to understand the effect of lysozyme on a commercial scale basis.

4.2 LITERATURE CITED

Bartowsky, E.J. & Henschke, P.A., 2004. The 'buttery' attribute of wine-diacetyl-desirability, spoilage and beyond – Review article. Int. J. Food Microbiol. 96, 235-252.

Beelman, R.B., Govin, A. & Keen, R.M., 1977. A new strains of *Leuconostoc oenos* for induced malo-lactic fermentation in eastern wines. Am. J. Enol. Vitic. 28, 159-165.

Britz, T.J. & Tracey, R.P., 1990. The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos*. J. Appl. Bacteriol. 68, 23-31.

Campos, F.M., Couto, J.A. & Hogg, T.A., 2003. Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. J. Appl. Microbiol. 94, 167-174.

Carreté, R., Vidal, M.T., Bordons, A. & Constantí, M., 2002. Inhibitory effect of sulphur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. FEMS Microbiol. Lett. 211, 155-159.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H., 1985. Practical implications of malolactic fermentation. A review. Am. J. Enol. Vitic. 36, 290-301.

Davis, C.R., Wibowo, D.J., Lee, T.H. & Fleet, G.H., 1986. Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. Appl. Environ. Microbiol. 51, 539-545.

Davis, C.R., Wibowo, D., Fleet, G.H. & Lee, T.H., 1988. Properties of wine lactic acid bacteria: their potential enological significance. Am. J. Enol. Vitic. 39, 137-142.

Du Toit, M. & Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - A review. S. Afr. J. Enol. Vitic. 21, Special Issue, 74-96.

Edwards, C.G., Haag, K.M., Semon, M.J., Rodriguez, A.V., Mills, J.M., 1999. Evaluation of processing methods to control the growth of *Lacobacillus kunkeei*, a micro-organism implicated in sluggish alcoholic fermentations of grape musts. S. Afr. J. Enol. Vitic. 20, 11-18.

Gao, Y-C., Zhang, G., Krentz, S., Darius, S., Power, J. & Lagarde, G., 2002. Inhibition of spoilage lactic acid bacteria by lysozyme during wine alcoholic fermentation. Aus. J. of Grape and Wine Res. 8, 76-83.

King, S.W. & Beelman, R.B., 1986. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. Am. J. Enol. Vitic. 37, 53-60.

Kunkee, R.E., 1991. Some roles of malic acid in the malolactic fermentation in wine making. FEMS Microbiol. Rev. 88, 55-72.

Lafon-Lafourcade, S., Carre, E. & Ribéreau-Gayon, P., 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl. Environ. Microbiol. 46, 874-880.

Lafon-Lafourcade, S., Geneix, C. & Ribéreau-Gayon, P., 1984. Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by yeast ghosts. Appl. Environ. Microbiol. 47, 1246-1249.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek, 76, 317-331.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud, A., 2000. The Microbiology of Wine and Vinifications. Handbook of Enology, vol. 1, John Wiley & Sons Ltd.

Stratiotis, A.L. & Dicks, L.M.T., 2002. Identification of *Lactobacillus* spp. isolated from different phases during the production of a South-African fortified wine. S. Afr. J. Enol. Vitic. 23, 14-21.

Vidal, M.T., Poblet, M., Constantí, M. & Bordons, A., 2001. Inhibitory effect of copper and dichlofluanid on *Oenococcus oeni* and malolactic fermentation. Am. J. Enol. Vitic. 52, 223-229.

Vivas, N., Lonvaud-Funel, A. & Glories, Y., 1997. Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. Food Microbiol. 14, 291-300.

Vivas, N., Augustin, M. & Lonvaud-Funel, A., 2000. Influence of oak wood and grape tannins on the lactic acid bacterium *Oenococcus oeni* (*Leuconostoc oenos*, 8413). J. Sci. Food Agric. 80, 1675-1678.

Wibowo, D., Fleet, G.H., Lee, T.H. & Eschenbruch, R.E., 1988. Factors affecting the induction of malolactic fermentation in red wines with *Leuconostoc oenos*. J. Appl. Bact. 64, 421-428.

APPENDIX

APPENDIX

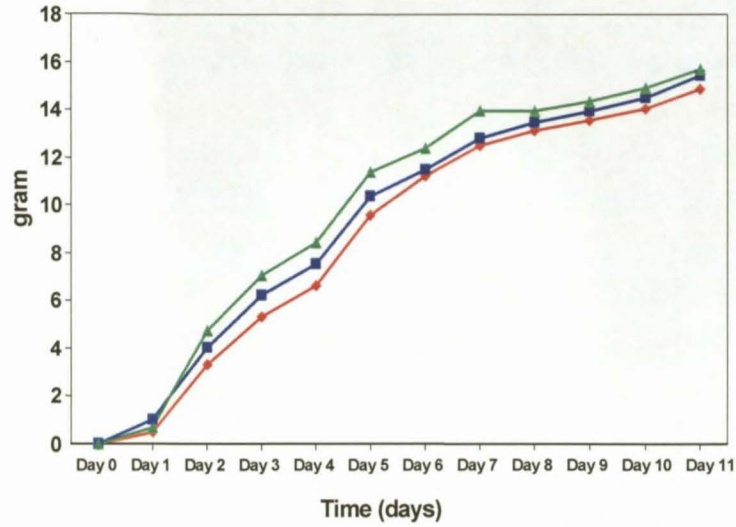


Figure 3.2 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with S. cerevisiae VIN 13 and *Lactobacillus nagelii* ATCC 700692^T at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

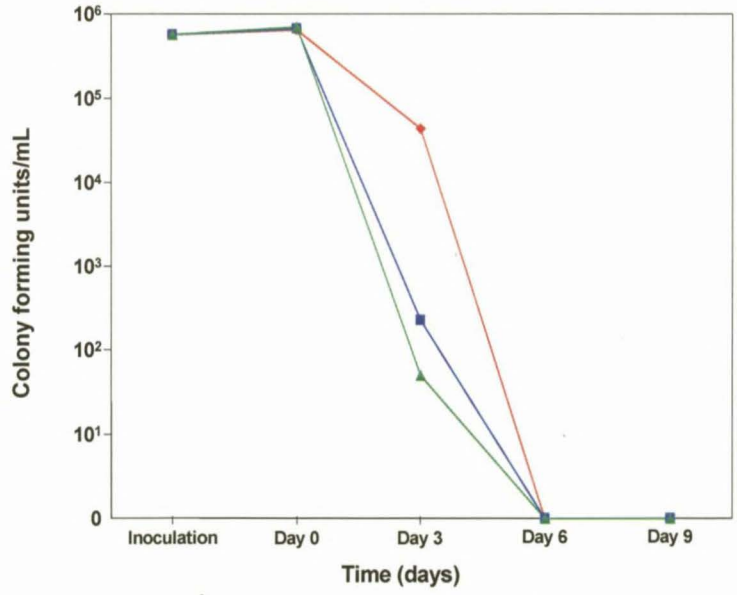


Figure 3.3 The effect of lysozyme on the growth of *Lactobacillus plantarum* LMG 13556. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

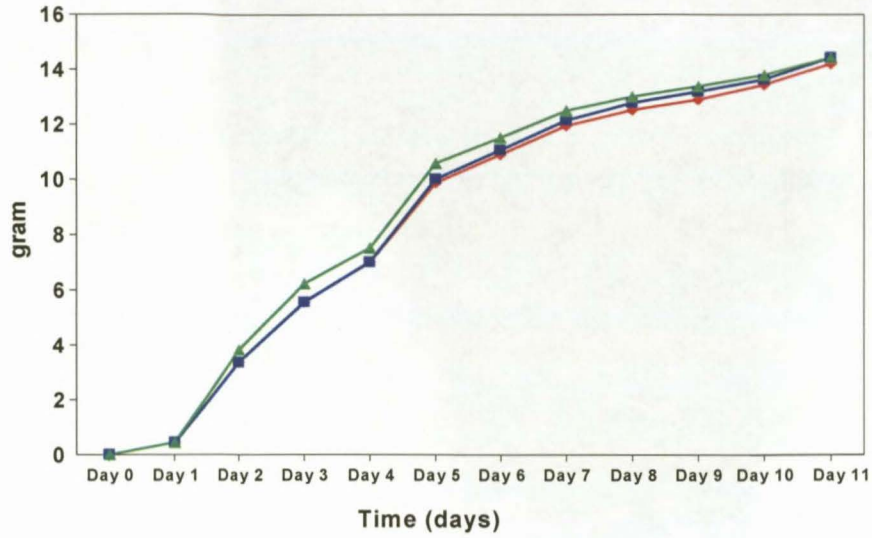


Figure 3.4 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus plantarum* LMG 13556 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

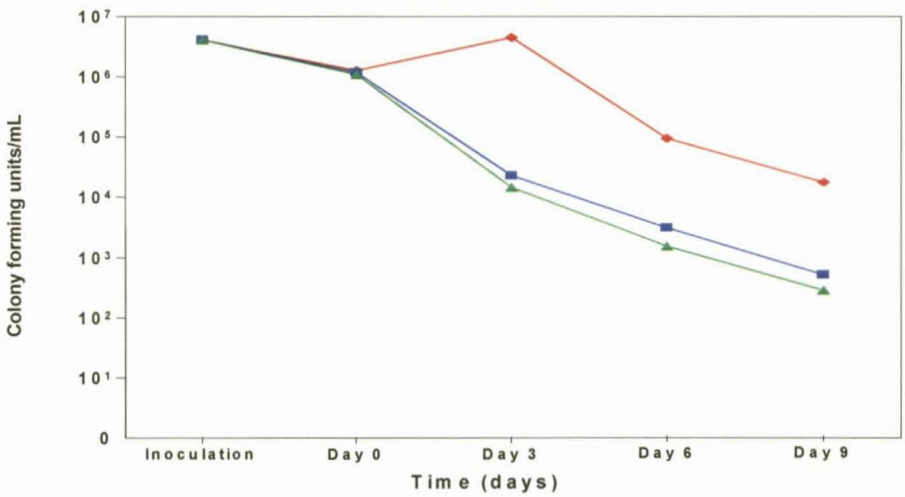


Figure 3.5 The effect of lysozyme on the growth of *Lactobacillus sakei* subsp. *sakei* LMG 13558^T. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

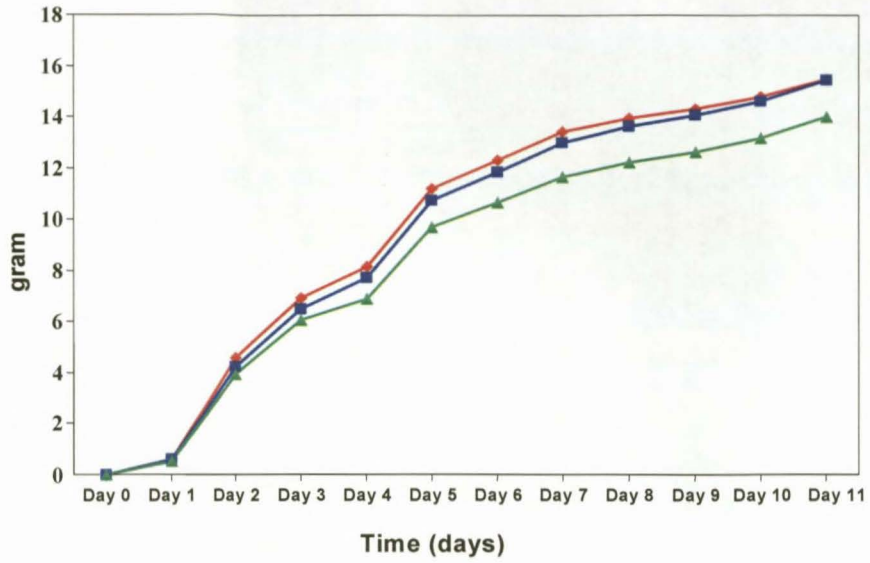


Figure 3.6 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus sakei* subsp. *sakei* LMG 13558^T at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

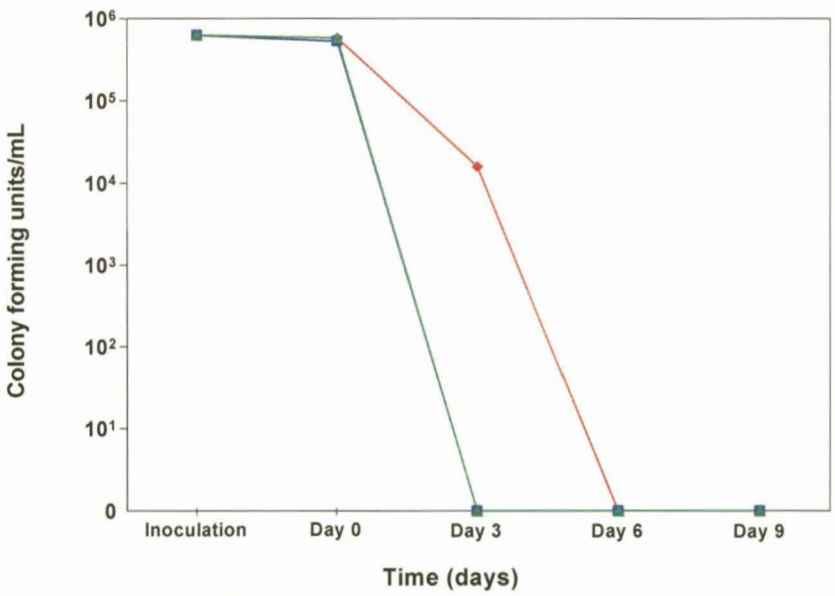


Figure 3.7 The effect of lysozyme on the growth of *Lactobacillus fermentum* LMG 13554. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

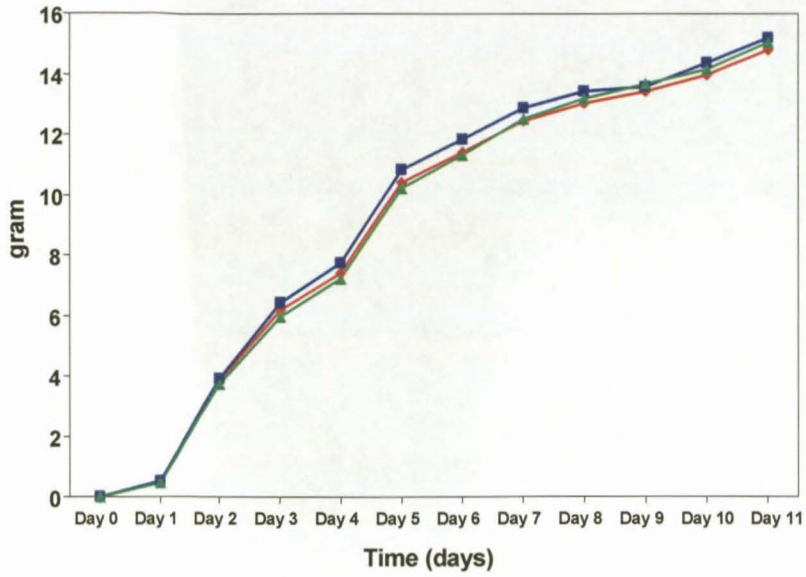


Figure 3.8 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus* fermentum LMG 13554 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme

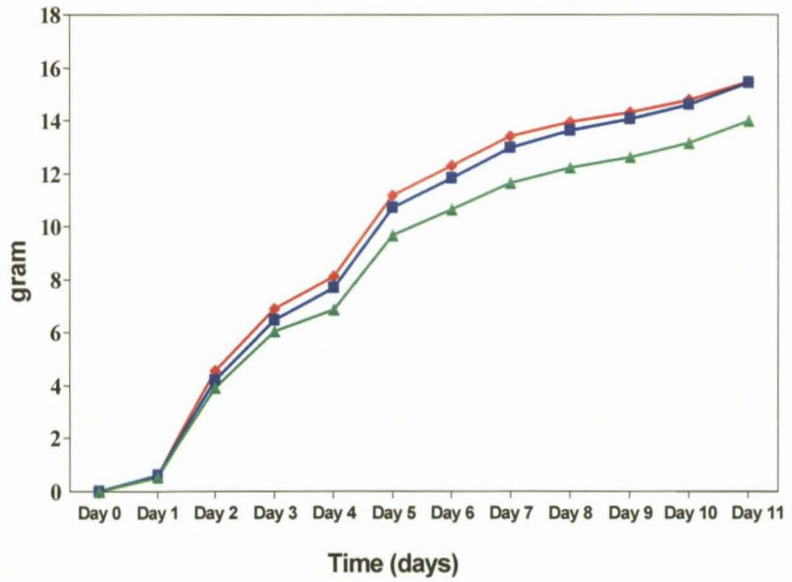


Figure 3.10 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus pentosus* DSM 20314^T at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

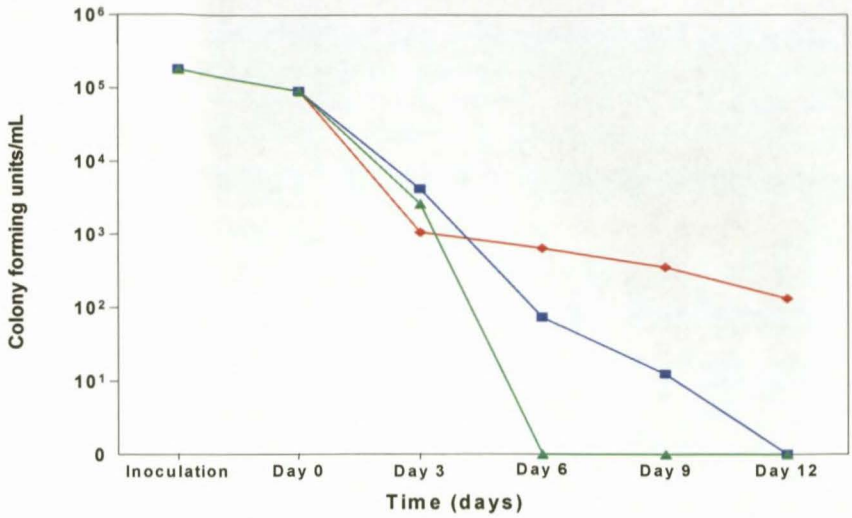


Figure 3.11 The effect of lysozyme on the growth of *Lactobacillus paracasei* DSM 20314^T. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

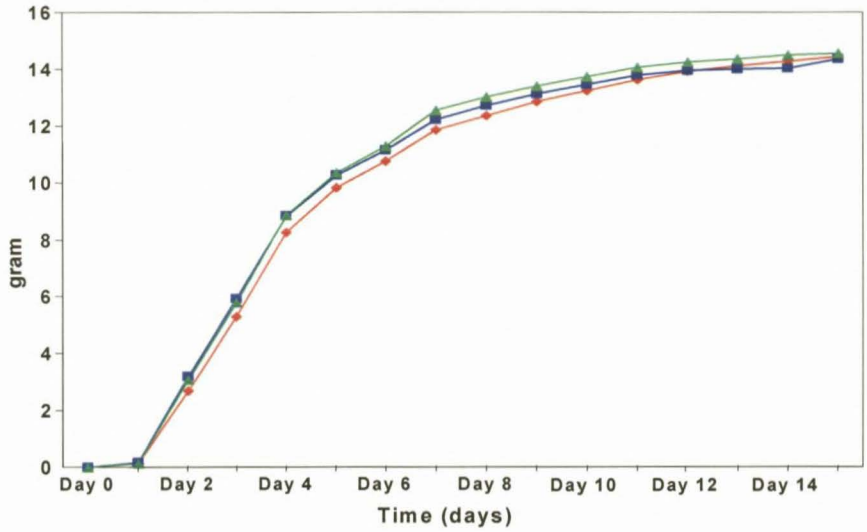


Figure 3.12 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus paracasei* DSM 20314^T at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

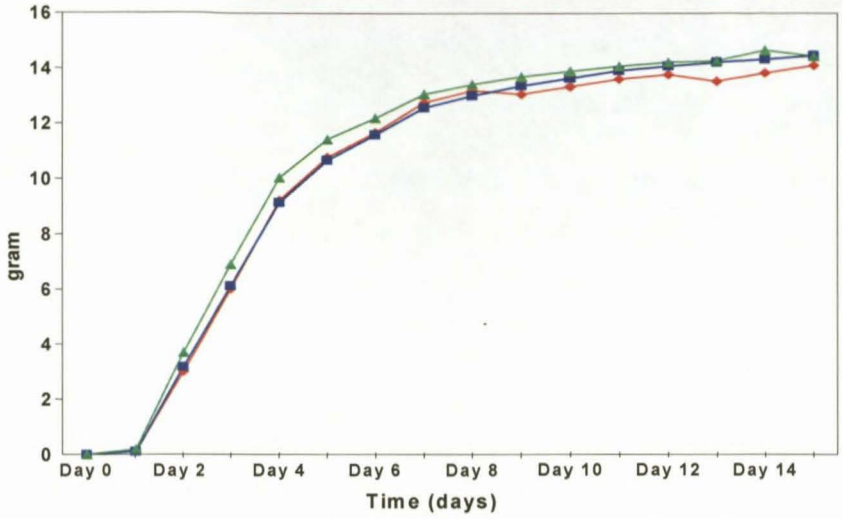


Figure 3.14 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus buchneri* DSM 20057^T at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

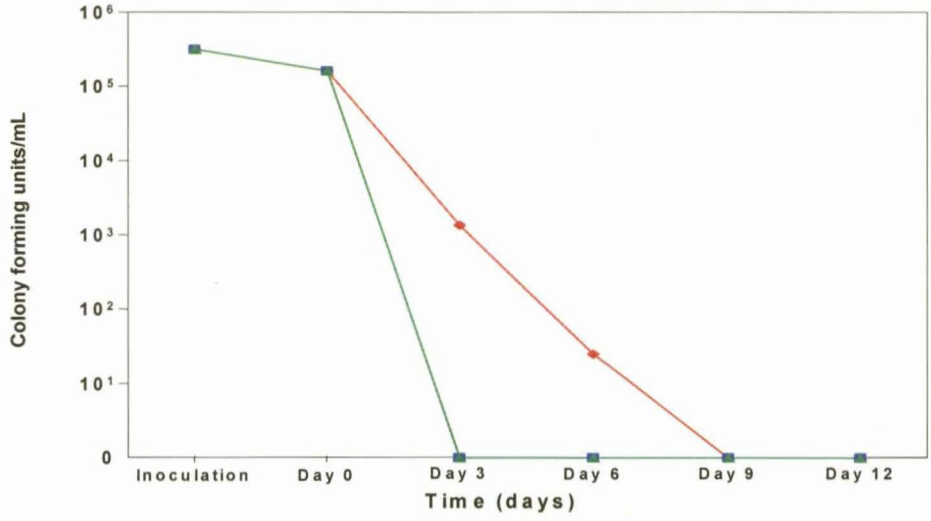


Figure 3.15 The effect of lysozyme on the growth of and *Pediococcus acidilactici* PAC 1.0. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

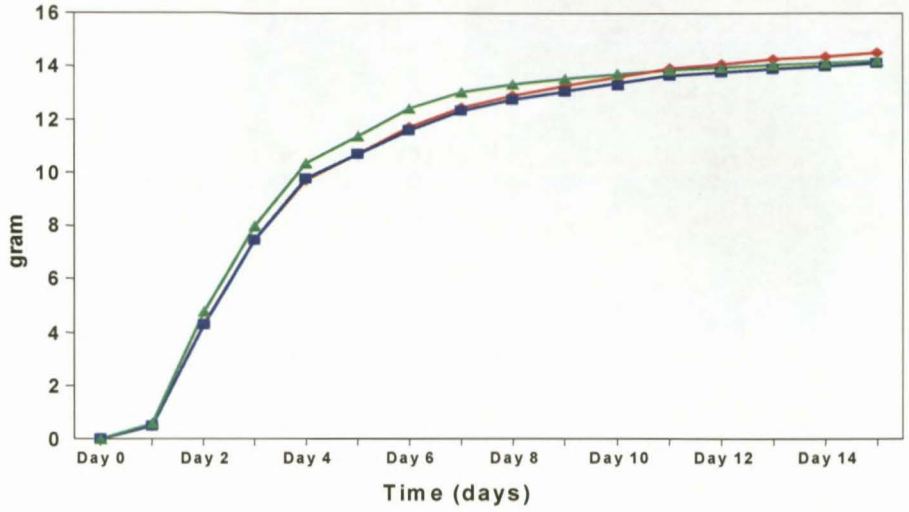


Figure 3.16 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Pediococcus acidilactici* PAC 1.0 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

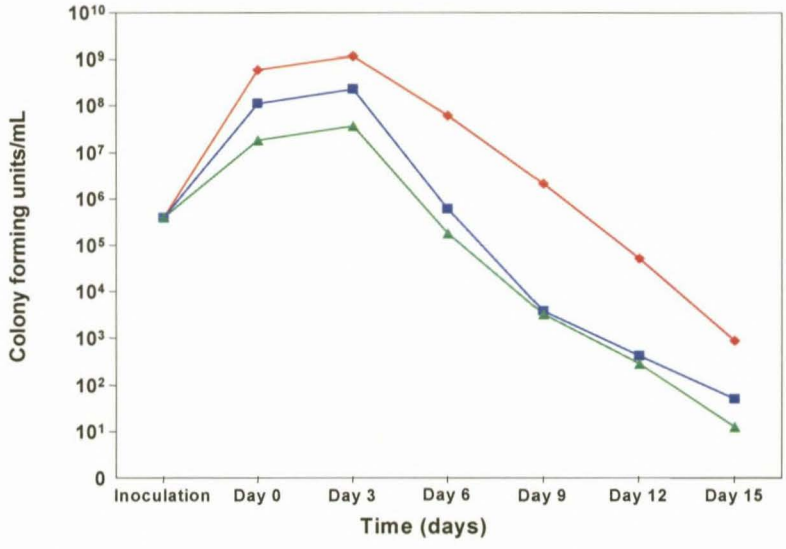


Figure 3.19 The effect of lysozyme on the growth of *Lactobacillus paracasei* wine isolate # 84. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

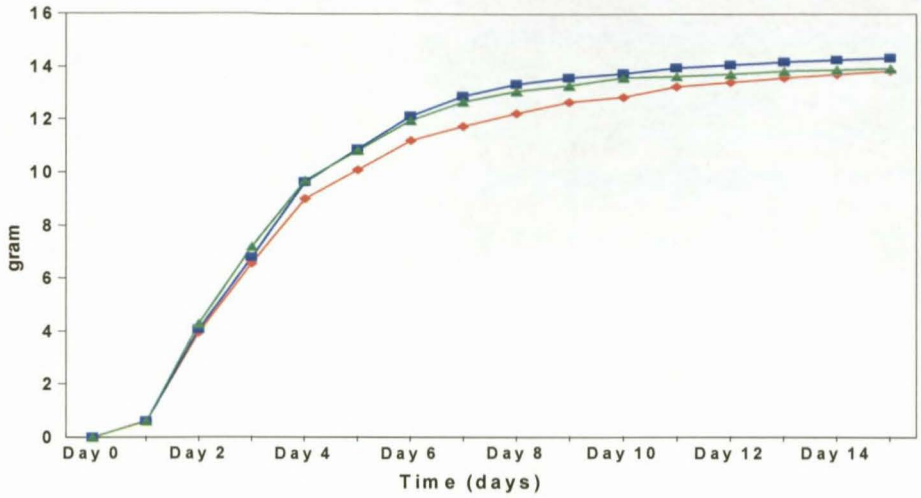


Figure 3.20 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus paracasei* wine isolate # 84 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

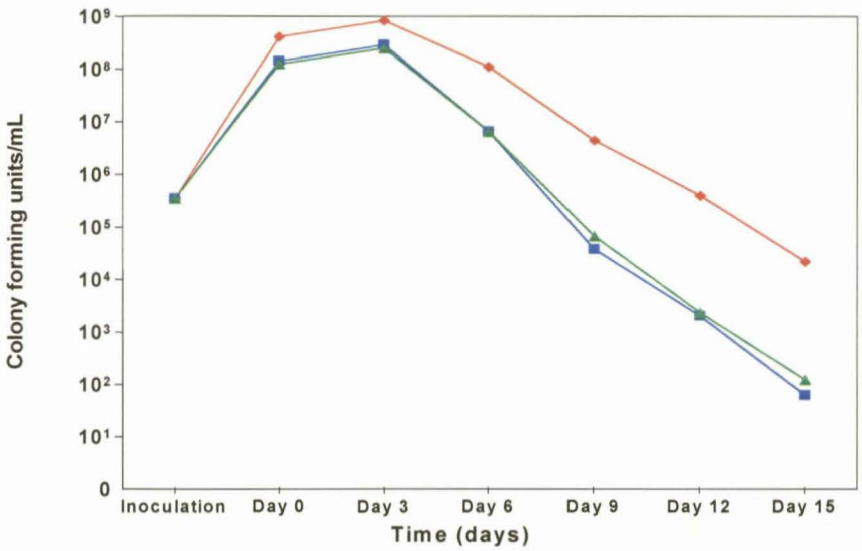


Figure 3.21 The effect of lysozyme on the growth of *Lactobacillus plantarum* wine isolate # 14. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

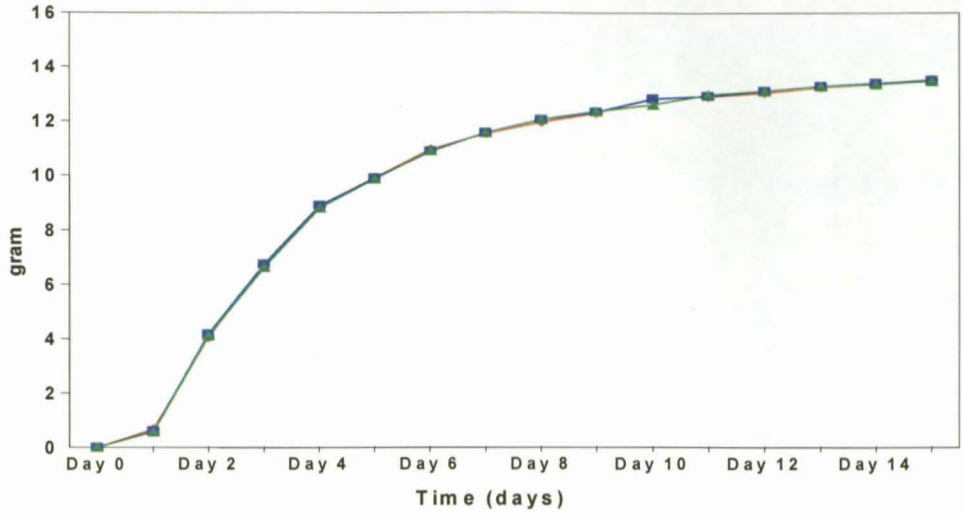


Figure 3.22 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus plantarum* wine isolate # 14 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

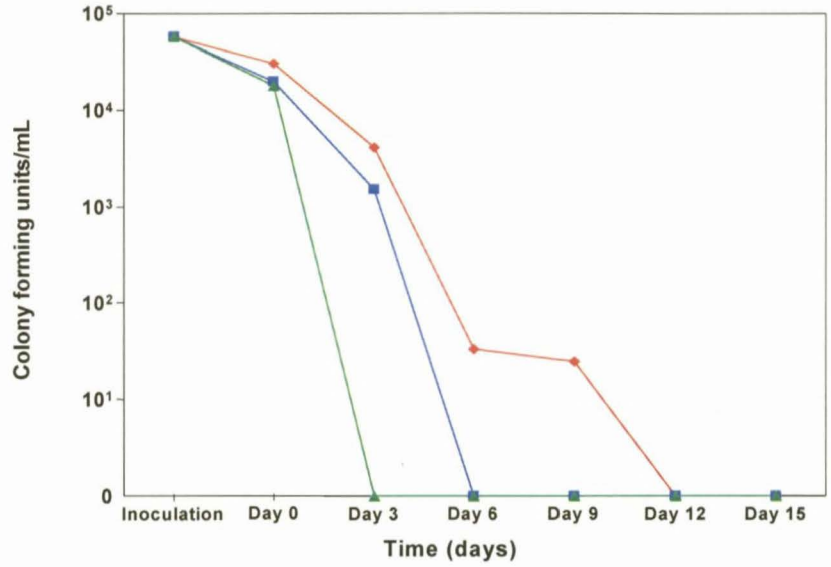


Figure 3.23 The effect of lysozyme on the growth of *Lactobacillus pentosus* wine isolate # 42. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

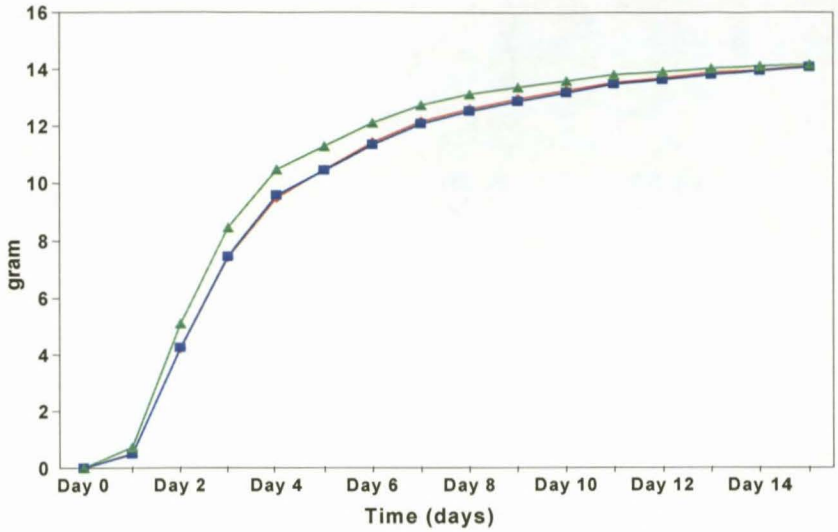


Figure 3.24 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus pentosus* wine isolate # 42 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

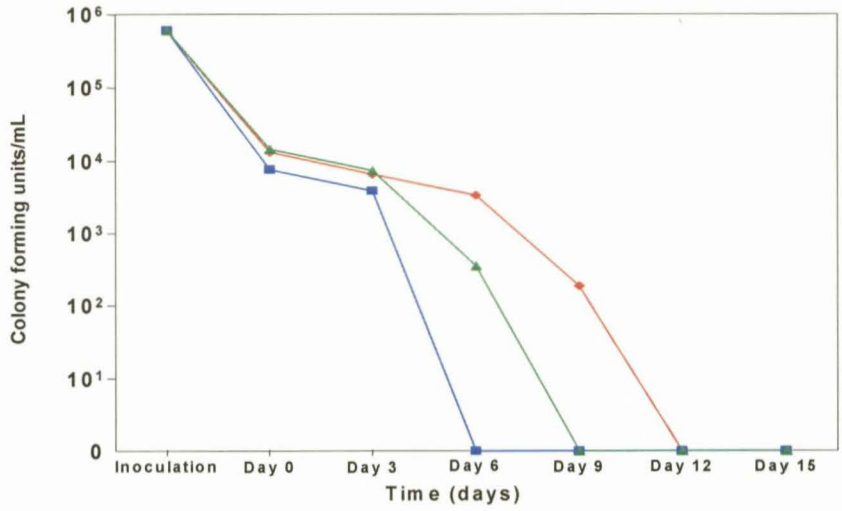


Figure 3.25 The effect of lysozyme on the growth of *Pediococcus acidilactici* wine isolate # 118. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

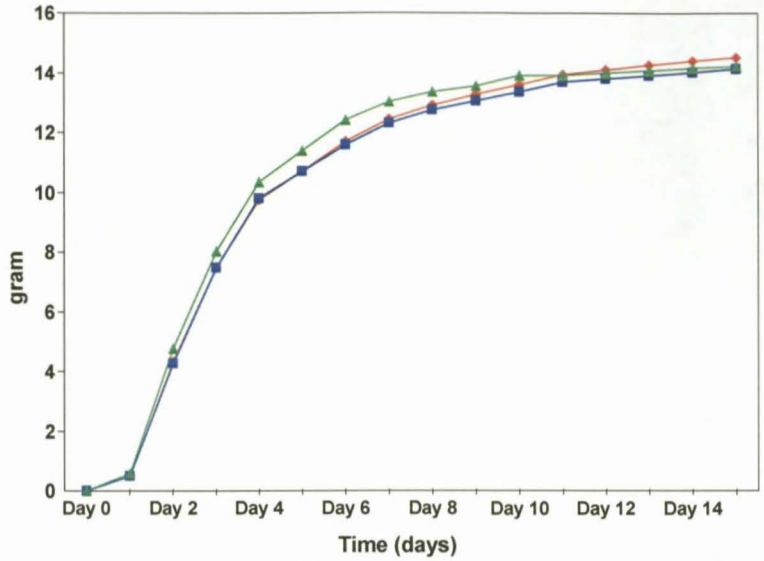


Figure 3.26 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Pediococcus acidilactici* wine isolate # 118 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

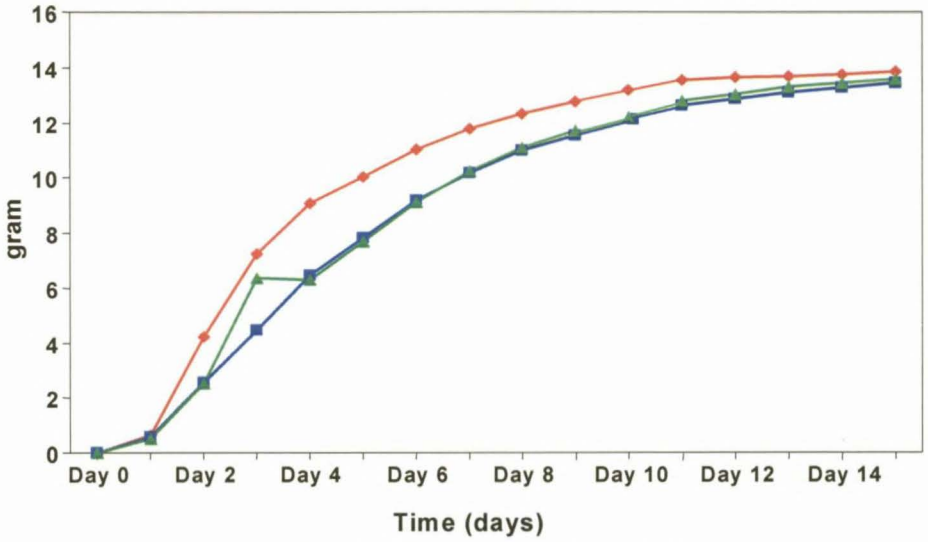


Figure 3.28 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus brevis* wine isolate # 81.1 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme

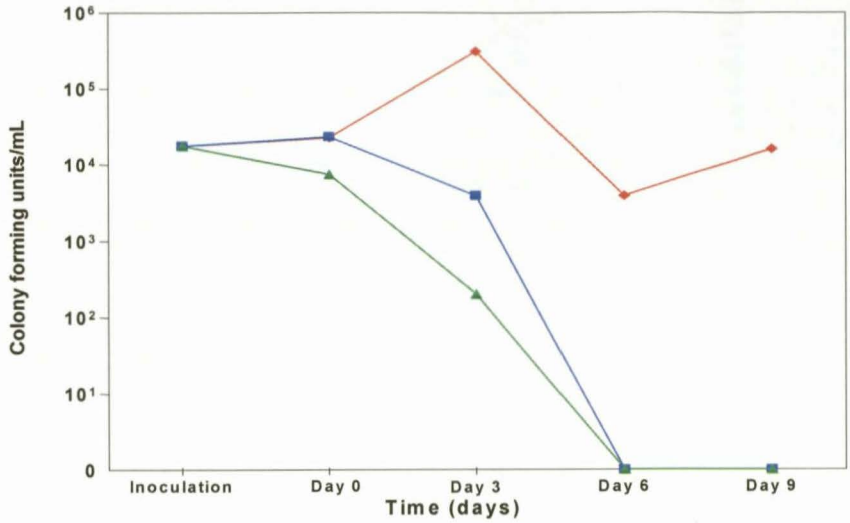


Figure 3.31 The effect of lysozyme on the growth of *Lactobacillus plantarum* wine isolate # 50. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

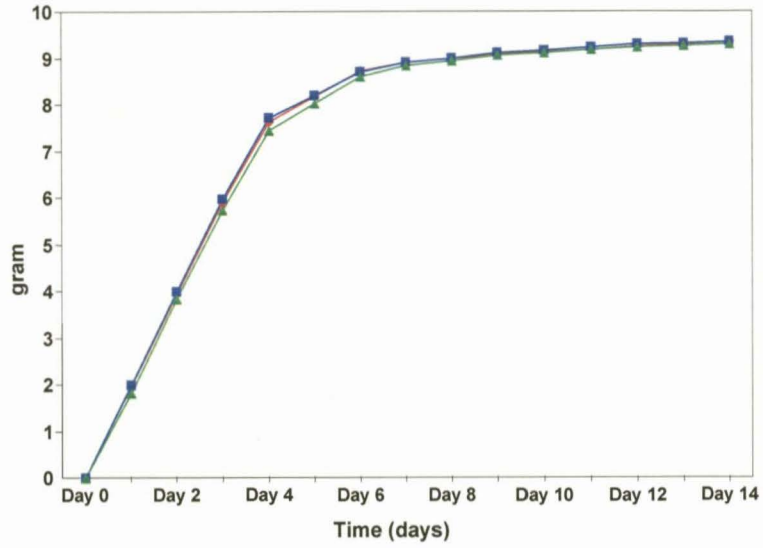


Figure 3.32 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus plantarum* wine isolate # 50 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

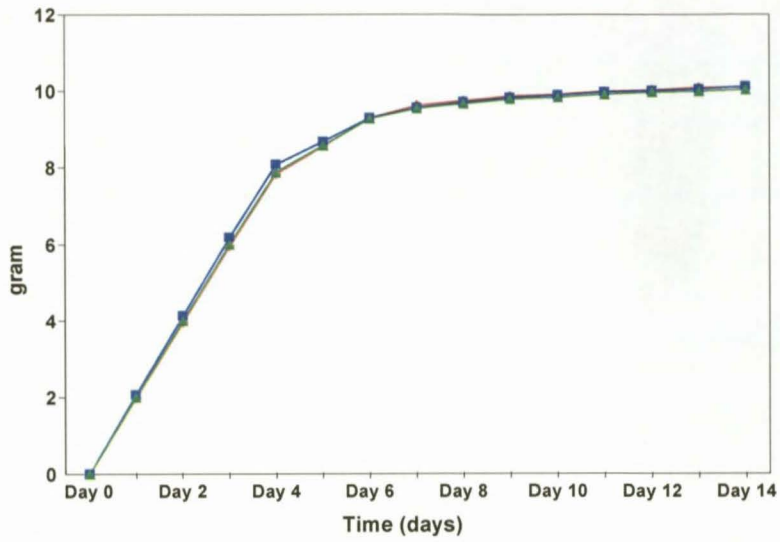


Figure 3.34 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus paraplantarum* wine isolate # 101 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

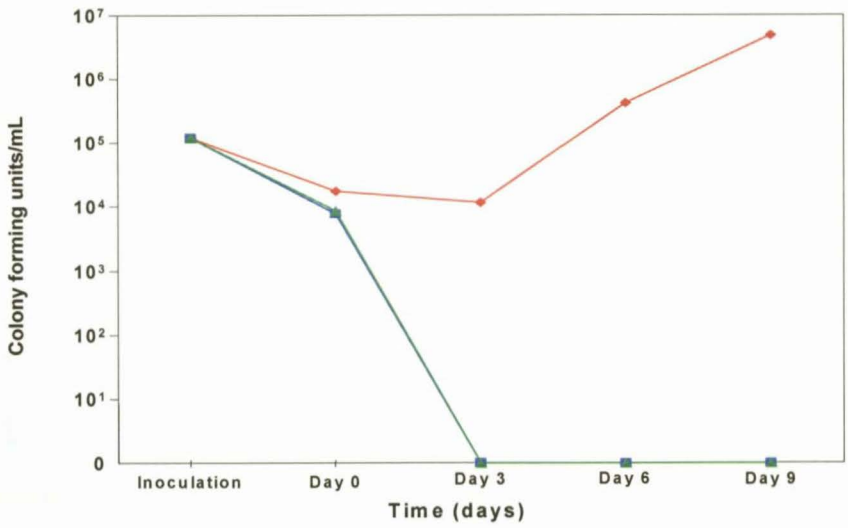


Figure 3.35 The effect of lysozyme on the growth of *Lactobacillus paracasei* wine isolate # 54. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

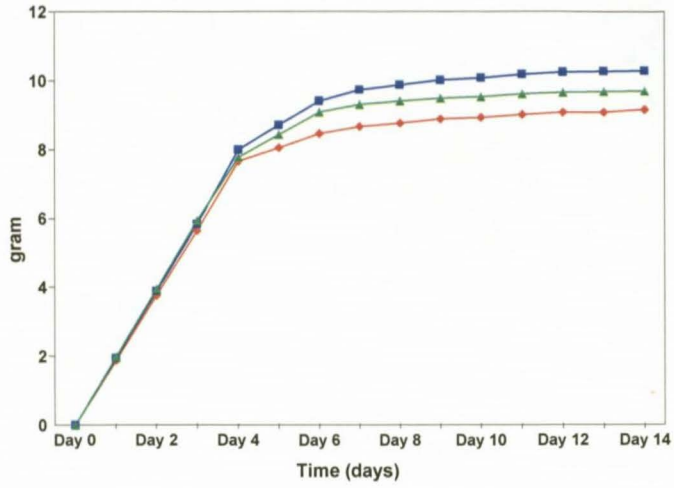


Figure 3.36 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus paracasei* wine isolate # 54 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

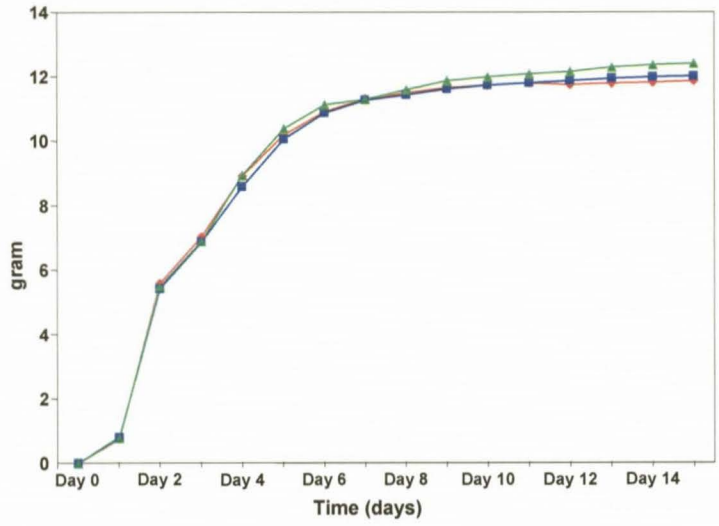


Figure 3.40 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus brevis* wine isolate J23 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

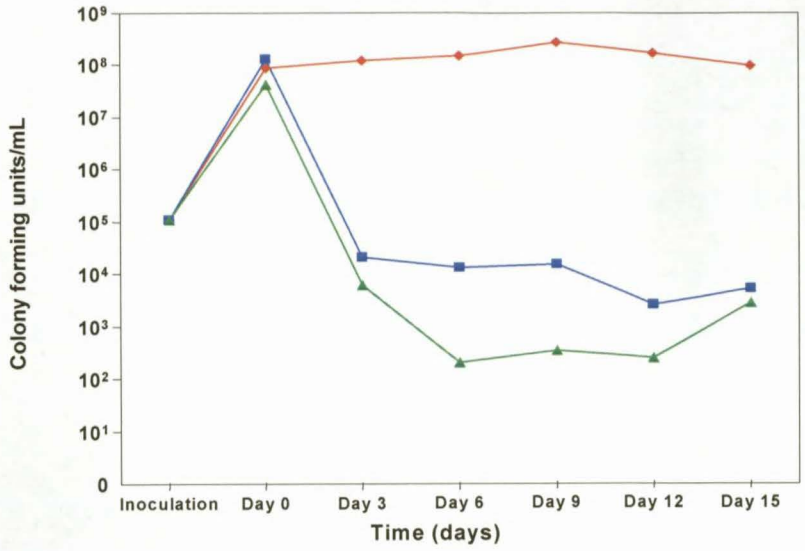


Figure 3.41 The effect of lysozyme on the growth of *Lactobacillus pentosus* wine isolate K22. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

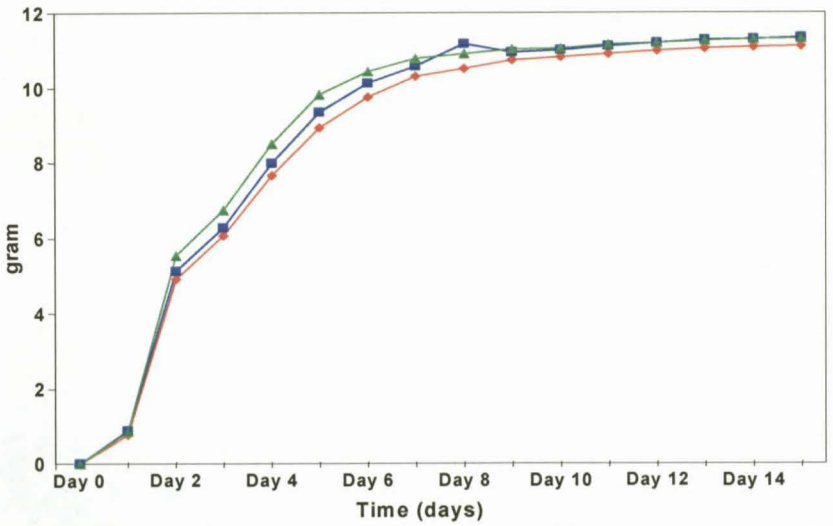


Figure 3.42 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus pentosus* wine isolate K22 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

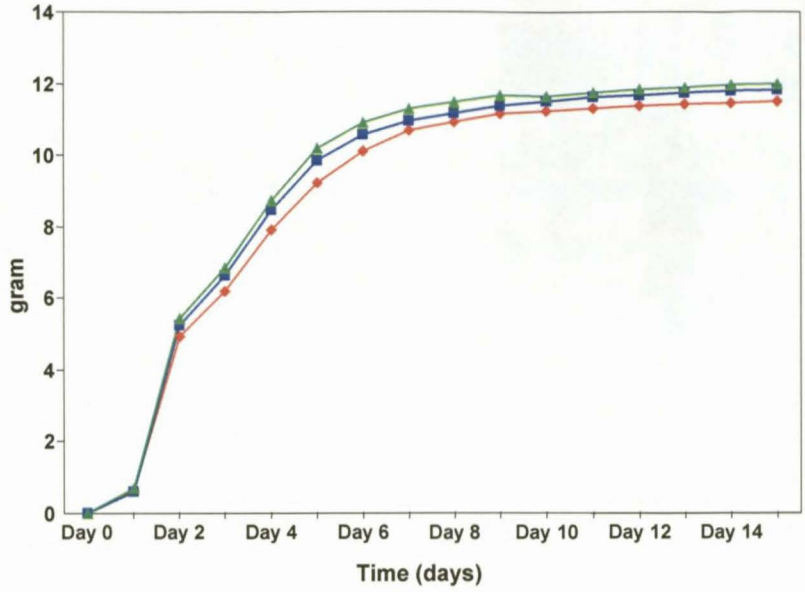


Figure 3.46 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with S. cerevisiae VIN 13 and *Lactobacillus plantarum* wine isolate K57 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

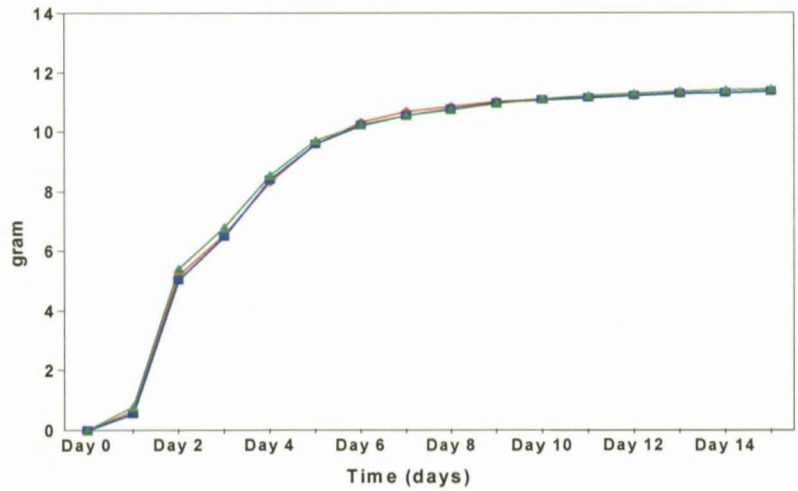


Figure 3.48 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus paracasei* wine isolate L43 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

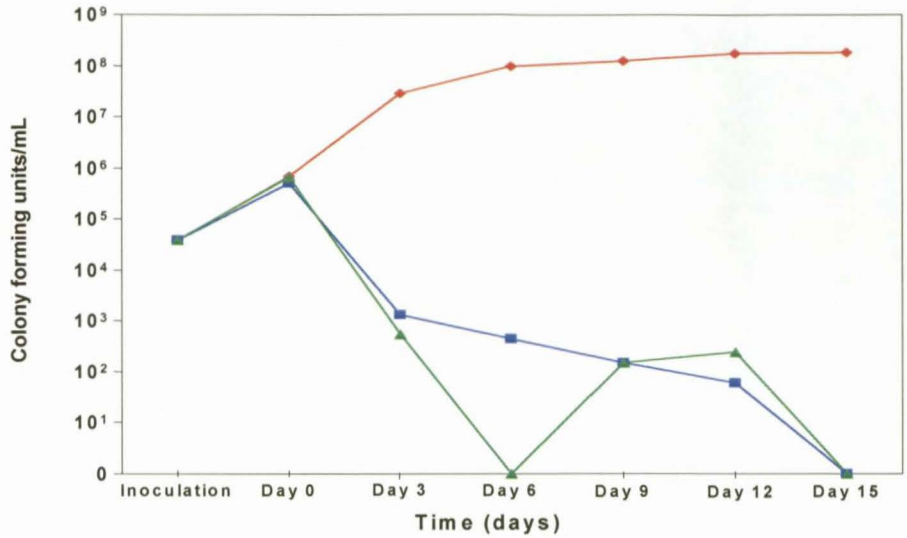


Figure 3.49 The effect of lysozyme on the growth of *Lactobacillus hilgardii* wine isolate M52. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

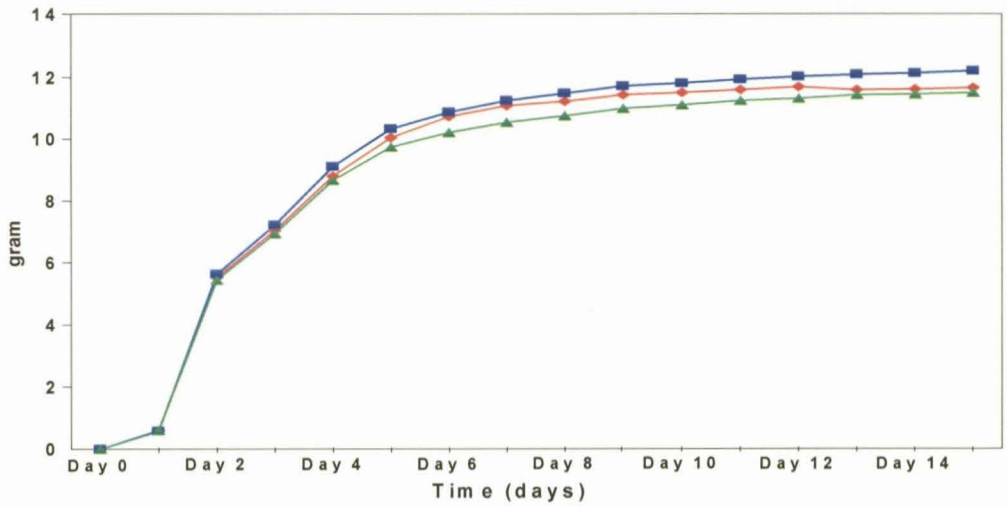


Figure 3.50 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus hilgardii* wine isolate M52 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

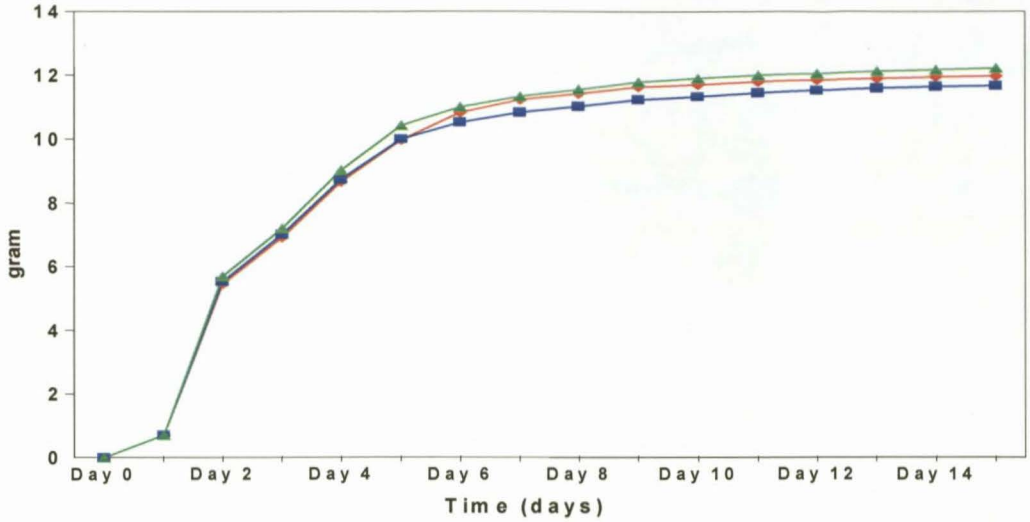


Figure 3.52 The accumulated CO_2 loss (in grams/150mL) of Chenin blanc juice inoculated with *S. cerevisiae* VIN 13 and *Lactobacillus vermiforme* wine isolate W16 at different lysozyme concentrations over time. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

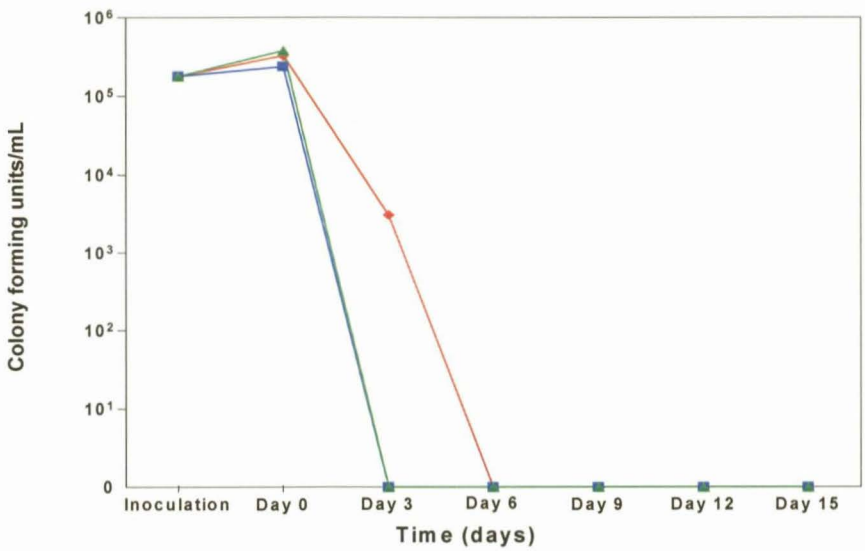


Figure 3.53 The effect of lysozyme on the growth of *Leuconostoc mesenteroides* wine isolate #5. ♦ Control; ■ 250 mg/L lysozyme; ▲ 500 mg/L lysozyme.

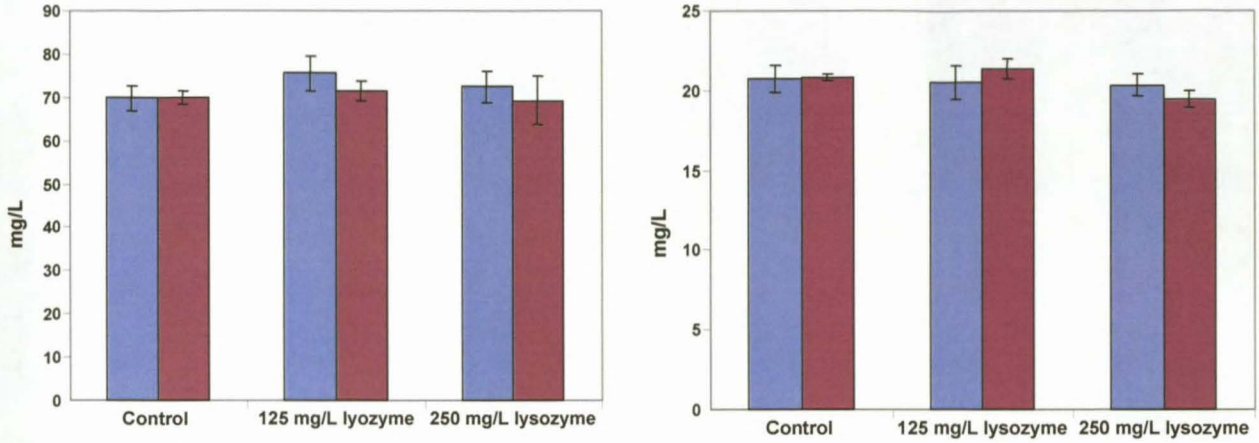


Figure 3.62. Ethyl acetate concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

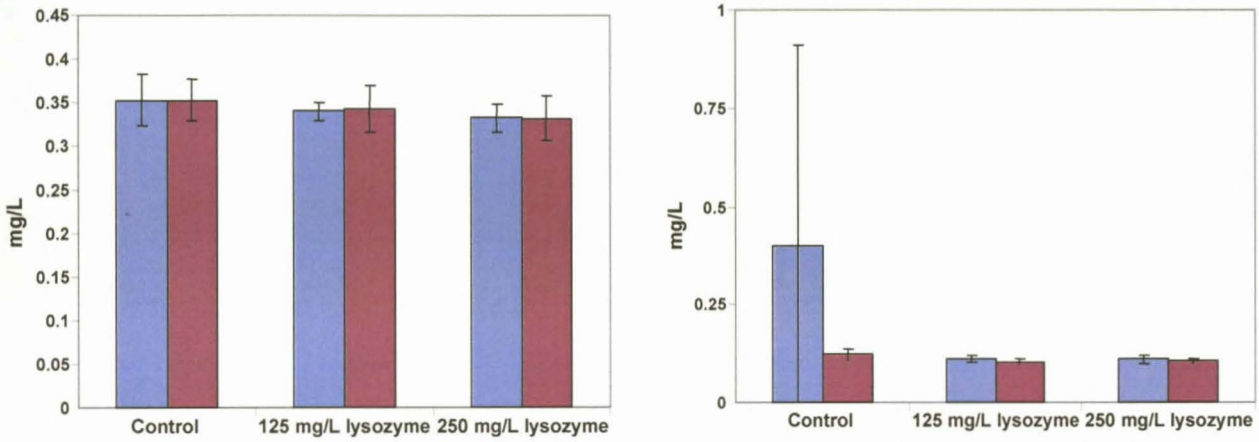


Figure 3.63. Ethyl hexanoate concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

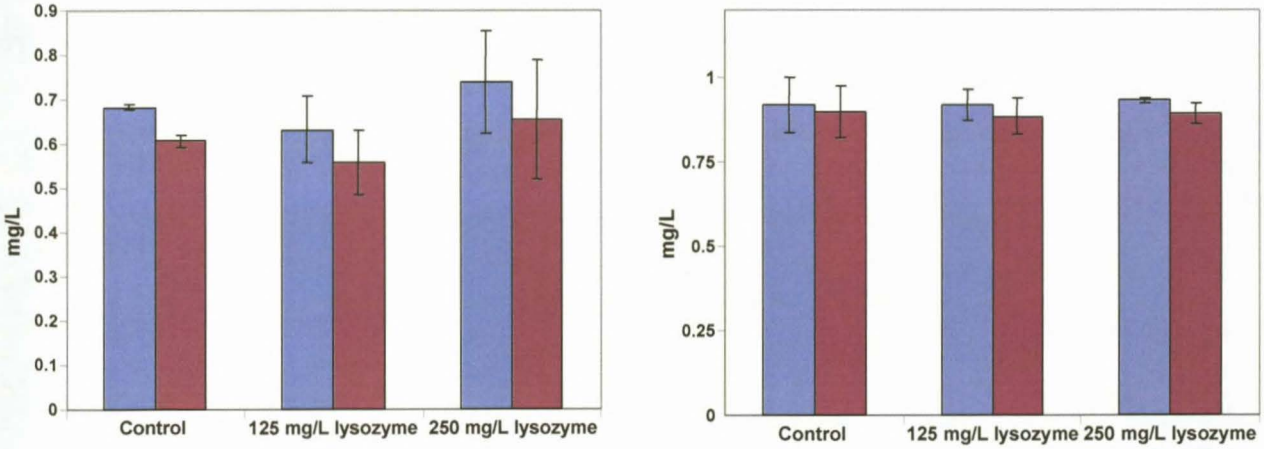


Figure 3.65. Hexanol concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

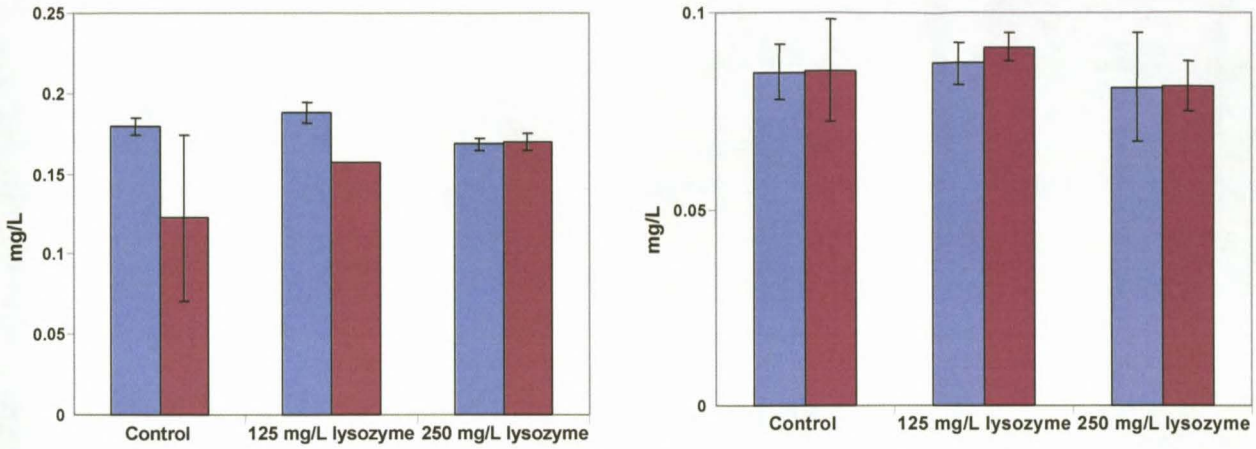


Figure 3.66. Hexyl acetate concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

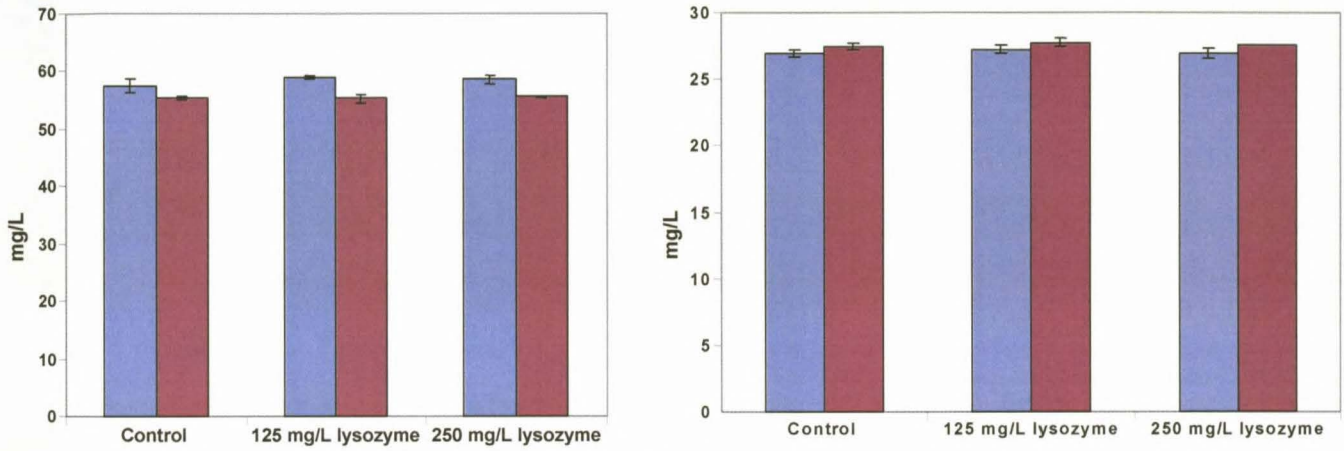


Figure 3.68. iso-Amyl alcohol concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

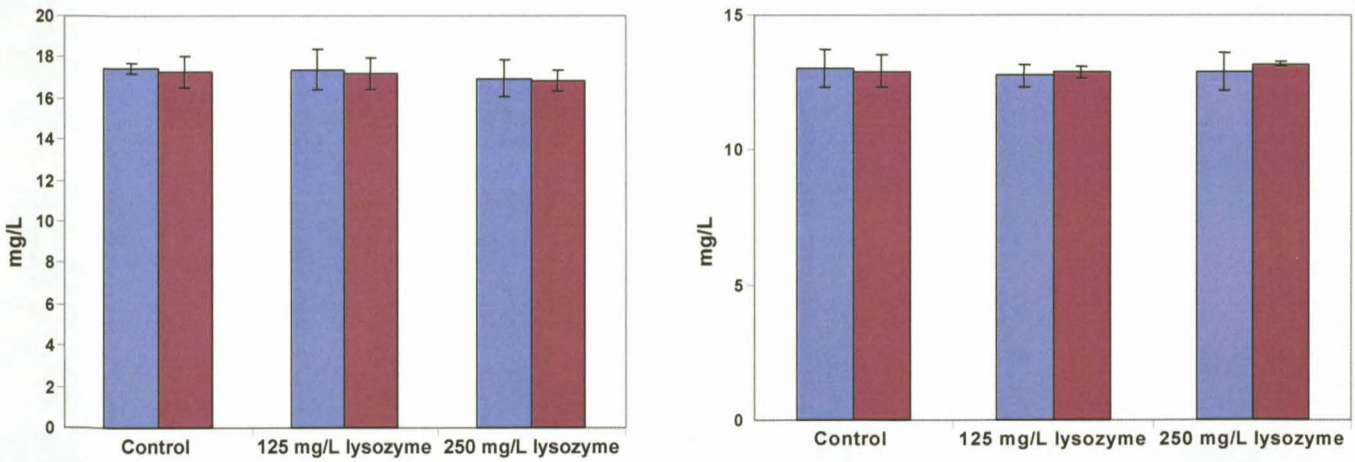


Figure 3.69. iso-Butanol concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

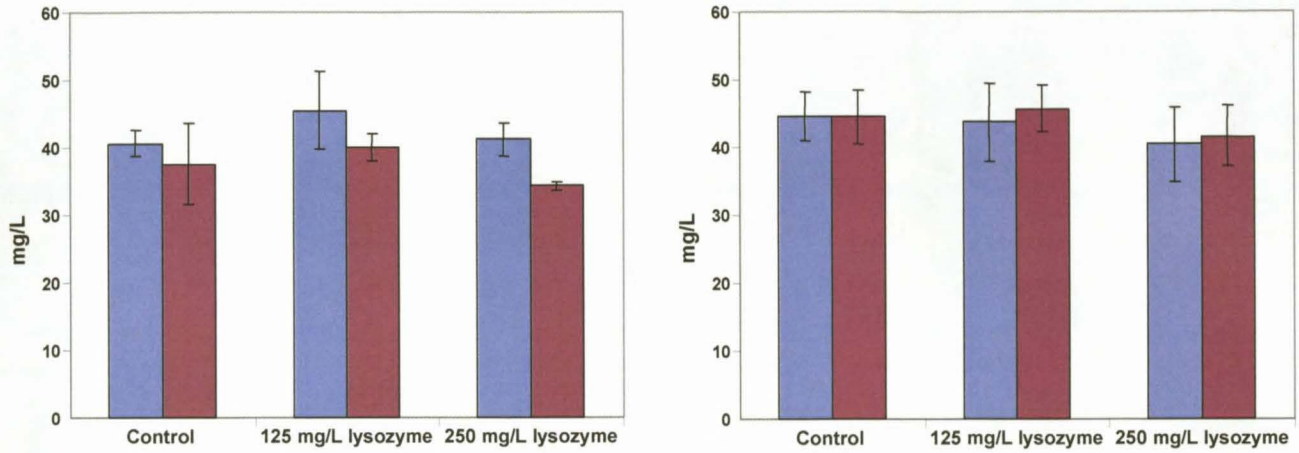


Figure 3.70. Methanol concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.

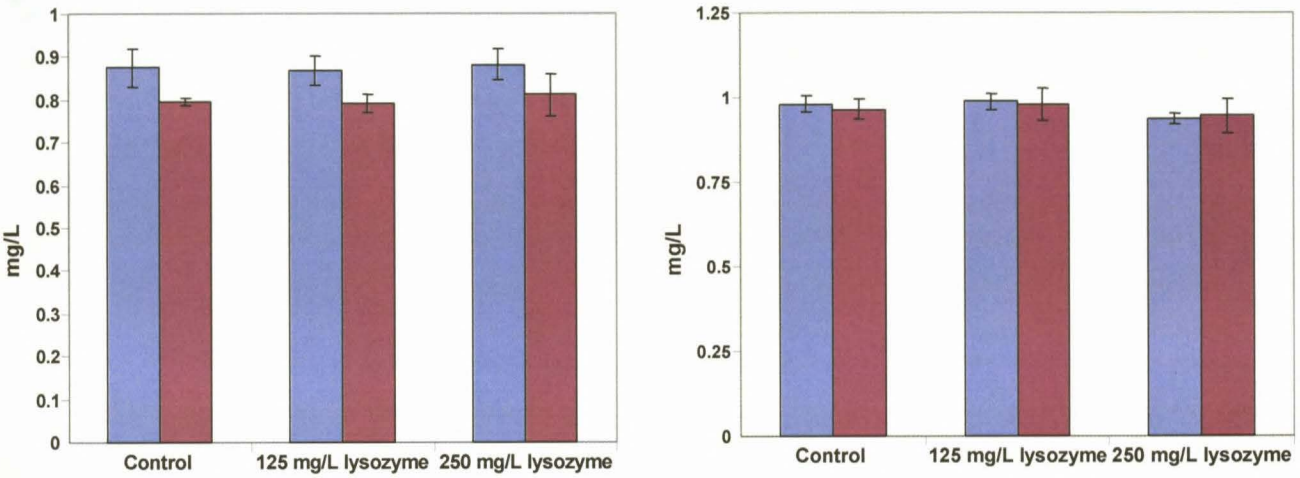


Figure 3.71. n-Butanol concentrations in Pinotage (left) and Shiraz (right). ■ Post-MLF; ■ End of AF.